

**SirT1 regulates bone mass *in vivo* through regulation of
osteoblast and osteoclast differentiation**

by

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B.S. Molecular, Cell, and Developmental Biology
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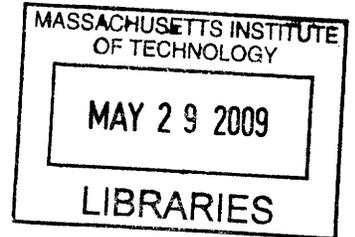
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ABSTRACT

As mammals age, osteoblast numbers decrease while osteoclast numbers increase, resulting in age-related or post-menopausal osteoporosis. SirT1, the mammalian orthologue of yeast Sir2, has been shown to be a negative regulator of PPAR γ and NF κ B, two transcription factors important for the differentiation of osteoblasts and osteoclasts, respectively. Here, we show that SirT1 is an important regulator of bone mass *in vivo*: SirT1 whole-body knockout mice display significant bone deficiencies that are associated with decreased osteoblast and increased osteoclast numbers. Further, osteoblast (ObKO) and osteoclast (OcKO) specific SirT1 knockout mice also show an osteoporotic phenotype that is associated with decreased osteoblasts in the ObKOs and increased osteoclasts in the OcKOs. In osteoblasts, we find that instead of targeting PPAR γ , SirT1 interacts with and increases the transactivation potential of Runx2. In osteoclasts, SirT1 inhibits differentiation largely through repression of the p65/RelA subunit of NF κ B. Finally, we show that calorie restriction (CR) results in increased bone mass that is associated with higher osteoblast and lower osteoclast numbers – the inverse phenotype of SirT1 knockout mice. We find CR induces expression and activity of SirT1 in bone, and that SirT1 knockout mice fail to show any increases in bone mass in response to CR. Our results therefore provide the first evidence that SirT1 is an important regulator of bone mass *in vivo*, possibly providing a link between bone remodeling, metabolism and osteoporosis.

Thesis Supervisor: Leonard Guarente
Title: Novartis Professor of Biology

DEDICATION

I would like to dedicate this thesis to my undergraduate mentor, Dr. Eri Srivatsan. The time I spent in his lab has largely shaped me into the scientist I am now. Thank you, Dr. Sri.

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Chapter 1

Introduction

Regulation of Aging in Eukaryotes

IS AGING AMENABLE TO SCIENTIFIC STUDY?

Everything that lives, dies. Alternatively, everything that lives, ages and then dies.

Although these two concepts are intuitive to all of us, they differ in one important regard – the rate of death. The latter idea depicts a mortality rate that increases with age (**Figure 1A**), while the former depicts one in which death can occur with equal probability at any age (**Figure 1B**). Empirically, we know that death is much more likely with advanced age (and all of us know that our grandparents are more likely to die than our parents). And though it may seem strange to say it, old age is the single greatest risk factor for death.

Evolutionary theory of aging versus experimental observations

All this begs the question: what exactly is aging and what causes it? Evolutionary biology tells us that aging is actually the concomitant breakdown of multiple biological systems due to the declining influence of natural selection in the post-reproductive phase of life. Since most animals in the wild do not survive into old age (instead dying from predation, starvation or other extrinsic factors), it has been argued that there has been very little pressure to select for alleles that would confer any benefits later in life (reviewed in Kirkwood, 2005). Consistent with this, the forces of natural selection are known to be strongest early in life. As a result, the post-reproductive phase of life is generally associated with a multiplicity of phenotypes that we associate with aging; resulting largely because natural selection no longer acts to maintain them. Interestingly, the rules of natural selection are thought to also give rise to a phenomenon termed, antagonistic pleiotropy, where genes that provide substantial benefits at early life might actually be detrimental during later life (Williams, 1957). For the above reasons, evolutionary theory has

argued that aging is an inevitable byproduct of the rules of natural selection and is therefore not a regulated process amenable to scientific study.

In spite of these arguments, evidence began to emerge in the late twentieth century that challenged this widely held notion. First, it appeared that the mortality curves for wildly divergent organisms closely resembled each other, suggesting a commonality in the aging process that could potentially be studied in model organisms (**Figure 1C**). Secondly, while aging itself is not regulated, it was clear the rate of aging could be regulated. This was best exemplified by the vastly disparate lifespans of closely related species, such as those of the rodent family: a typical rat lives around 5 years, while the average naked-mole rat lives approximately 30 years (Buffenstein, 2005). Even within the same species, dramatically different aging rates can exist. Take for example the case of the honey bee: even though the queen bee is genetically identical to the workers bees, she lives on average ten times longer (Corona et al., 2007).

However, the strongest case was made by calorie restriction (CR) - a simple environmental intervention that extends the lifespan of nearly all organisms studied. CR is simplicity at its best: by simply reducing the food source by a moderate amount (in mammals this equates to 30-40% below ad libitum), one can extend both average and maximum lifespan of virtually any organism (Koubova and Guarente, 2003). Conversely, there also exist a series of premature aging diseases in mammals (such as Hutchinson-Guilford Syndrome and Werner Syndrome) that appear to accelerate many features of the aging process, including osteoporosis, cataracts, alopecia and premature death (Brosh and Bohr, 2007). These data together strongly suggest that the rate of aging is likely a regulated process and therefore amenable to scientific inquiry. The race to identify the genes responsible for longevity was on.

Regulation of aging through the insulin signaling pathway

In 1988, the first evidence for the genetic regulation of longevity emerged: Friedman and Johnson published their seminal paper showing that hypomorphic mutations in the age-1 gene (now known to be PI(3) Kinase) extended lifespan of the roundworm *C. elegans* (Friedman and Johnson, 1988). This simple observation implicated what has become the best characterized aging pathway in worms - the insulin/insulin-like growth factor 1 (IGF-1)-like endocrine system. Later studies would show that mutations in other components of the pathway also increased lifespan, including DAF-2 (which encodes an insulin/IGF-1 receptor homologue), and its downstream targets (Kenyon et al., 1993; Kimura et al., 1997; Paradis et al., 1999). Further inquiry revealed that life extension through this pathway was absolutely impingent on nuclear translocation of DAF-16 (a FOXO family transcription factor), delineating a clear genetic signaling pathway that regulated aging in *C. elegans*. It also demonstrated that the aging process was in fact regulated and amenable to genetic manipulation (Kenyon et al., 1993).

Interestingly, the insulin pathway is not merely important for worms: mutations in the insulin/IGF-1 pathway have recently been shown to extend lifespan in higher organisms such as flies and even mice (Kenyon, 2005). Even the yeast SCH9 protein, which shows significant homology to the worm homologues AKT-1 and AKT-2, has been shown to influence aging (Fabrizio et al., 2001). Since yeast lack hormone signaling, this remarkable finding suggests that evolution has selected for certain genes to modulate the aging process in a more generalized, context-dependent manner.

Sir2, an NAD dependent histone deacetylase implicated in longevity

The next breakthrough came in 1995 when a second group of researchers set out to find additional longevity associated genes, this time in the budding yeast *S. cerevisiae*. Taking advantage of a correlation between stress resistance and longevity, Kennedy and co-workers

identified a series of mutants that were both resistant to starvation and had prolonged lifespan (Kennedy et al., 1995). One of these strains carried a mutation in Sir4 (Silent Information Regulator) which redirected the silencing complex of Sir2, Sir3 and Sir4 away from telomeres and mating type loci to the ribosomal RNA locus (rDNA) in the nucleolus (Kennedy et al., 1997). Subsequent experiments demonstrated that Sir2 was the critical component of the complex that was required for the observed longevity phenotype: Sir2 normally silenced the rDNA locus even without the other Sir proteins (Smith and Boeke, 1997); Sir2 contained the catalytic domain responsible for the silencing (Bryk et al., 1997); and overexpression of Sir2 alone increased lifespan in yeast, while deletion of Sir2 shortened it (Kaeberlein et al., 1999). Reminiscent of the insulin signaling pathway, it once again appeared that single genes could dramatically influence the rate of aging of an organism. However, two important questions remained: how did silencing at the rDNA lead to increased lifespan and what exactly was the enzymatic function of Sir2?

The first question was answered in 1997 when Sinclair and Guarente showed that the formation of extrachromosomal rDNA circles was a cause of aging in yeast (Sinclair and Guarente, 1997). The rDNA locus in yeasts contains 100-200 tandemly repeating copies of genes coding for the large and small rRNAs (35S and 5S rRNA) that are required for ribosomal assembly. Due to the repetitive nature of the locus, homologous recombination occurs between adjacent rDNA repeats, which results in the formation of extrachromosomal circular DNAs (ERCs). Although this is a relatively rare event, two properties of ERCs lead to their exponential expansion once formed: first, ERCs can replicate independently during S-phase since they contain an origin of replication; and second, ERCs tend to preferentially remain with the mother cells during cell division and thus do not segregate evenly to the newly formed 'young' daughter

cell (Sinclair and Guarente, 1997). As a result, even a few ERCs can eventually give rise to an amount of extraneous genetic material that rivals the rest of the yeast genome. This massive amount of superfluous DNA results in nucleolar instability and saps the nuclear machinery required for basic processes such as transcription and DNA replication. This results in the decreased fitness of the mother cell that is observed with aging.

The causal role of ERCs in aging was validated when the exogenous addition of a single ERC to a young yeast cell was sufficient to reduce its lifespan (Sinclair and Guarente, 1997). Conversely, genetic mutations that reduce the formation of ERCs by decreasing the recombination rate at the rDNA locus also resulted in increased lifespan (Defossez et al., 1999). And in 2000, the final piece of the puzzle was solved: it was shown that Sir2 catalyzed a novel NAD (nicotinamide adenine dinucleotide) dependent histone deacetylation reaction that led to heterochromatic silencing of the rDNA. This silencing reduced the frequency of homologous recombination and thereby repressed formation of ERCs, resulting in increased longevity (Imai et al., 2000).

Intriguingly, the coupling of an energetically important molecule such as NAD with Sir2 enzymatic function provided a startling molecular link between metabolism, epigenetic silencing and longevity. And as will be discussed later, life extension via Sir2 would transcend its humble origins in yeast – Sir2 overexpression would be later shown to extend lifespan of higher organisms such as worms and flies (even though these organisms are largely post-mitotic). Thus, at the dawn of the new millennium, after a mere decade of study, it appeared that the rate of aging was in fact a highly regulated process, amenable to control by even single genes.

CALORIE RESTRICTION (CR) AND LIFE EXTENSION: THE SIR2 CONNECTION

Calorie restriction (CR), which involves moderately reducing food intake below ad libitum (AL) levels but not to the point of malnutrition, has been known to extend the lifespan of virtually all organisms tested. This includes (but is not limited to) yeast, worms, flies, rodents and likely primates (Koubova and Guarente, 2003). The fact that CR works in such widely different organisms suggests that it has been selected for early in evolution, possibly as a survival mechanism for times of food scarcity. The idea goes as follows: organisms that can delay reproduction and redirect resources to the soma during unfavorable times, such as those associated with the natural boom/bust cycle, stand a better chance of surviving and reproducing when conditions are more favorable. This idea of hunkering down during harsh times is not just a theory – it is vividly exemplified by organisms such as microbes and worms which can form highly stress resistant alternate forms such as spores and dauer larvae, respectively. Not surprisingly, the DAF-2 signaling pathway that mediates dauer formation in worms, also promotes longevity (Kenyon, 2005). These observations, therefore, suggest that proteins that are keenly attuned to the environmental conditions of the organism might serve as ideal candidates for mediating a pro-longevity response.

Early theories: is CR a regulated or passive process?

In mammals, CR has been shown to forestall many of the diseases associated with aging. For example, mice fed 30-40% below ad libitum levels are more resistant to the development of cardiovascular disease, diabetes, autoimmune disease, kidney disease, as well as a number of neurodegenerative and neoplastic disorders (Koubova and Guarente, 2003). In addition, CR also leads to a healthy metabolic profile associated with longevity, including lower blood glucose levels, increased insulin sensitivity, and reduced inflammatory cytokines. Other well documented physiological changes associated with CR are reduced fertility, lower core body temperature, and

changes in the neuroendocrine system including lower levels of growth hormone, thyroid stimulating hormone, IGF-1, gonadotropins; and increased glucocorticoids, catecholamines and glucagons. Importantly, these numerous changes are associated with a highly robust extension in both average and maximal lifespan. This life extension can reach approximately 50%, or in human terms, an extra 30-40 years. This begs the question: how does CR mediate these powerful and wide reaching effects?

Early theories attempted to attribute these effects as passive or indirect consequences of a slowing in metabolism. For example, one of the first widely held theories argued that the benefits of CR were largely due to decreased production of reactive oxygen species (ROS) associated with decreased food intake (Koubova and Guarente, 2003). This idea held sway since oxidative damage to DNA, RNA, protein and lipids were known to increase during aging, and enzymes that reduced ROS accumulation extended lifespan of model organisms. Additionally, studies confirmed that that oxidative damage was in fact lower in CR animals, providing further support for the theory. However, the theory relied on one critical parameter as a cornerstone of its argument - namely that metabolism actually slowed during CR. As a result, when studies showed that respiration actually increased in yeast and worms during CR, this theory was largely debunked (McCarter et al., 1985; Lin et al., 2002). Another leading theory that attributed increased protein turnover as the underlying cause of CR induced longevity also met a similar fate. However, molecular studies soon began to emerge that suggested that CR might in fact be a highly regulated process.

Sir2, a mediator of CR induced life extension

The studies outlining the molecular mechanism behind CR have their humble beginnings in the budding yeast, *S. cerevisiae*. This organism divides asymmetrically to produce a fully

'young' daughter cell and an 'aged' mother cell. The mother cell can divide approximately twenty times before it senesces and stops dividing, while the newly formed daughter cell has a full replicative potential (Guarente and Kenyon, 2000). Sinclair and colleagues showed in 1997 that aging of the mother cell was in part due to the accumulation of toxic extrachromosomal rDNA circles (ERCs) brought about by homologous recombination between the highly repetitive rDNA genes present in the yeast nucleolus (Sinclair and Guarente, 1997). Genetic interventions that reduced the formation of these ERCs were shown as sufficient to extend lifespan.

Much like in all other organisms, CR can extend the lifespan of the aging mother yeast cell, in this instance by around 30%. In yeast, CR usually entails simply reducing the glucose concentration of the media from 2% to 0.5%, though a newer, more extreme regimen has brought this concentration further down to 0.05% (**Figure 2**) (Lin et al., 2002; Kaerberlein et al., 2005). Under the more moderate form of CR, the growth rate of yeast remains the same, even though the cells are under a partial state of energy limitation. As a result, the cells shift their metabolic strategy: instead of fermenting glucose to produce ATP, they divert the carbons to the TCA cycle. This of course makes sense since cells can harvest much more ATP through respiration than fermentation. The importance of this metabolic shift is underscored by the fact that it is both required and sufficient for life extension by CR (Lin et al., 2002). But how does this metabolic shift affect lifespan?

An early clue came when it was found that Sir2 mutants did not live longer under CR (Lin et al., 2000). Intriguingly, yeast strains genetically modified to have reduced NAD synthesis also did not live longer under CR, implicating the newly discovered NAD-dependent enzymatic activity of Sir2 (Lin et al., 2000). Next, CR was shown to activate Sir2 enzymatic function by two mechanisms: by increasing the NAD/NADH ratio in the cell and decreasing the levels of the

Sir2 inhibitor, nicotinamide (**Figure 2**) (Lin et al., 2004; Anderson et al., 2003). Further, CR failed to further extend lifespan of yeast overexpressing Sir2, suggesting the two were in the same pathway (Lin et al., 2000). Consistent with this, small molecule activators of Sir2 have also been shown to promote longevity in a manner similar to CR (and not in addition to CR) (Howitz et al., 2003). All in all, these experiments point towards Sir2 as a key mediator of the life-extending properties of CR in yeast, in part by reducing the formation of toxic ERCs.

And although ERC accumulation has now been shown to be a yeast specific cause of aging (no evidence of ERC accumulation exists in higher organisms), Sir2 overexpression has nonetheless been shown to extend the lifespan of other organisms such as worms and flies. Thus, it again appears that specific genes has been selected to regulate longevity, though likely in context-dependent manners.

THE SIRTUIN FAMILY OF DEACETYLASES/ADP-RIBOSYLTRANSFERASES

In yeast, Sir2 is the founding member of the Sirtuin family of deacetylases consisting of SIR (silent information regulator) 1 – 4, as well as HST (homologues of Sir2) 1 – 4. The Sirtuins are responsible for silencing at the yeast mating-type loci and telomeres, whereas Sir2 and HST2 are unique in that they can also silence the rDNA locus (Lin et al., 2000; Lamming et al., 2005). Sir2 orthologues can be found throughout evolution in organisms ranging from archaea to mammals, underscoring the important function they play (which has been preserved over billions of year). And although their molecular targets have evolved with the rise of the more complex metazoans, their key role in longevity has not.

Regulation of longevity in diverse organisms

One of the most intriguing findings in Sirtuin biology came when the overexpression of Sir2 was found to also extend the lifespan of worms (Lin et al., 2000; Tissenbaum and Guarente,

2001). Since yeast and worms diverged more than a billion years ago, this finding suggested that the ability of Sir2 to regulate lifespan also existed in their last common ancestor; as well as in all descendants, including mammals (**Figure 3**) (Guarente and Picard, 2005). This theory was confirmed when it was demonstrated that Sir2 overexpression also extended the lifespan of flies. Further, resveratrol, a small molecule agonist of Sir2, also extends the lifespan of worms, flies, and even the shortlived vertebrate fish species, *Nothobranchius furzeri* (Rogina and Helfand, 2004; Howitz et al., 2003; Wood et al., 2004; Valenzano et al., 2006). Intriguingly, life-extension by CR and resveratrol is not additive, and both require the presence of wildtype Sir2 (Wang and Tissenbaum, 2006; Greer and Brunet, 2009). Given the mounting evidence that Sir2 might be an important regulator of longevity, it begs the question: what do the mammalian Sirtuins do?

The mammalian Sirtuins

Mammals have seven Sirtuins (SirT1-7) which all contain a conserved 275 amino acid catalytic core domain and unique N-terminal and/or C-terminal sequences (**Figure 4A**) (Frye, 2000). Sirtuins compose a novel class of deacetylases (class III), which unlike class I and II, require NAD (and not water) as a cofactor in the deacetylation reaction. In a two step reaction, Sirtuins couple deacetylation of a lysine residue to the hydrolysis of NAD, giving rise to a deacetylated lysine, nicotinamide and O-acetyl-ADP-ribose as products (**Figure 4B**) (Imai et al., 2000). And although Sirtuins are classically thought of as deacetylases, there is mounting evidence that some family members can also catalyze a robust ADP ribosyltransfer reaction (**Figure 4B**). This actually should not be a tremendous surprise: before discovery of its deacetylase reaction, Sir2 was once thought to be a possible ADP-ribosyltransferase (since ADP-ribosylation is a reaction intermediate). It is important to note that regardless of the enzymatic

function, one molecule of NAD is always consumed per reaction. This association of NAD with Sirtuin activity has put the Sirtuins at the nexus of metabolism and transcriptional regulation.

Molecular phylogenetic analysis has divided the mammalian Sirtuins into four different classes (classes I – IV) (**Figure 4C**). SirT1-3 have been placed in class I, which share homology with the yeast Sirtuins and Sir2-related proteins in most eukaryotes. In this class, SirT1 shares the greatest homology with yeast Sir2 and HST1, and worm Sir-2.1; whereas SirT2 and SirT3 share homology with yeast HST2 and other fungal and protozoan Sirtuins. SirT4 resides in class II which includes Sirtuins from bacteria, insects, nematodes, mold fungus and protozoans; SirT5 is a member of class III Sirtuins which are distributed widely in all prokaryotes; and SirT6 and SirT7 are members of class IV which are specific only to metazoans, plants and vertebrates (Frye, 2000; Michan and Sinclair, 2007).

Interestingly, compared to their yeast counterparts, it appears that the mammalian Sirtuins have diversified in their function and cellular localization. For example, SirT1, SirT6 and SirT7 are the only nuclear Sirtuins, while SirT2 is found primarily in the cytoplasm and SirT3, SirT4 and SirT5 occur within the mitochondria (Guarente and Picard, 2005). Further, even the nuclear Sirtuins have distinct subnuclear localizations: SirT1 is associated with euchromatin, SirT6 is associated with heterochromatin, and SirT7 is found in the nucleolus. And even their molecular targets seem to have expanded: while yeast Sirtuins are primarily histone deacetylases, it appears that the majority of the targets for the mammalian Sirtuins are non-histone substrates (which makes particular sense for the non-nuclear Sirtuins).

Even in terms of activity, not of all the mammalian Sirtuins appear to be true deacetylases: while SirT1, SirT2, SirT3, SirT5 and SirT7 show only a deacetylase activity, SirT4 instead exhibits a robust ADP ribosyltransferase activity, and SirT6 shows both (Michan and

Sinclair, 2007). In fact, the only thing that the Sirtuins share in common is their highly conserved NAD-dependent enzymatic core domain and their ubiquitous expression in nearly all tissues. With their diverse characteristics, the mammalian Sirtuins are emerging as major regulators of a broad spectrum of cellular and physiological processes such as metabolism, inflammation, apoptosis, proliferation, differentiation and rDNA transcription.

SirT1, the mammalian orthologue of yeast Sir2

Based on sequence similarity, SirT1 is predicted to be the mammalian orthologue of yeast Sir2 and as a result has been the most extensively studied (Frye, 2000). Much like its yeast counterpart, SirT1 is localized in the nucleus, contains a robust NAD-dependent deacetylase activity, and can silence regions of the genome by deacetylating histones H1, H3 and H4 (Michan and Sinclair, 2007). However, unlike Sir2, SirT1 also has a number of non-histone targets. In this regard, SirT1 has been shown to modulate a diverse array of physiological processes by deacetylating key transcription factors in each pathway. And although whether SirT1 regulates longevity *per se* still remains an unanswered question, many of the physiological outputs of SirT1 overlap with that of CR, suggesting a potential link between the two. This hypothesis has gained support in recent years from multiple studies: for instance, SirT1 knockout mice do not respond to certain aspects of CR, such as increased activity and upregulation of metabolism (Chen et al., 2005; Boily et al., 2008); resveratrol treatment has been shown to recapitulate the transcriptional profile of CR in different tissues (Lagouge et al., 2006; Baur et al., 2006); and CR results in an increase in the NAD/NADH ratio, as well as expression of SirT1, in multiple tissues (Cohen et al., 2004). Below we examine the major processes SirT1 regulates and how these overlap with what is observed with CR.

Role in cell survival and stress resistance

One major pathway that SirT1 regulates is cell survival in response to genotoxic stress. SirT1 does this by interacting with various proteins involved in DNA damage repair, cell cycle regulation, and apoptosis. For example, SirT1 has been shown to deacetylate Ku70 (a DNA repair factor) and thereby inhibit Bax mediated apoptosis (Cohen et al., 2004). By deacetylating Ku70, SirT1 promotes cell survival by allowing Ku70 to sequester the pro-apoptotic Bax protein away from the mitochondria, where it would otherwise trigger apoptosis. Additionally, SirT1 is known to attenuate TGF β (transforming growth factor beta) dependent apoptosis by deacetylating and promoting the degradation of its downstream molecule, Smad7 (Kume et al., 2007). This has been shown to be protective against glomerular mesangial cell death that is associated with kidney disease. In an interesting correlation, CR has been shown to extend the lifespan of Fischer rats and C57BL/6J mice, both of which die predominantly from kidney disease.

Importantly, SirT1 is also a major regulator of master tumor suppressor and cell cycle proteins. For example, SirT1 deacetylates p53 and p73, and thereby represses their ability to induce apoptosis in response to DNA damage or oxidative stress (Vaziri et al., 2001; Luo et al., 2001). Consistent with this model, thymocytes from SirT1 knockout mice display p53 hyperacetylation upon DNA damage and increased sensitivity to ionizing radiation (Cheng et al., 2003). Recently, it has emerged that SirT1, p53 and HIC1 (hypermethylated in cancer 1) in fact form an intricate feedback loop that helps coordinate the cellular response to DNA damage (Chen et al., 2005). SirT1 and HIC1 form a complex which binds directly to the SirT1 promoter and represses its expression. p53 enters the fray by promoting the expression of HIC1 in response to genotoxic stress, thereby repressing expression of SirT1. Consistent with this negative feedback loop, p53 knockout mice show elevated SirT1 expression in many tissues.

Adding an additional layer of complexity, SirT1 also forms a similar loop with another cell cycle regulator, E2F1. In this case, E2F1 promotes transcription of SirT1, while SirT1 represses the apoptotic function of E2F1 (Wang et al., 2006). These complicated negative feedback loops are thought to exist in order to provide the cell with a sensitive mechanism to access the extent of cellular damage and decide accordingly whether to attempt repair or undergo apoptosis. By skewing the balance towards cell survival, SirT1 might potentially prevent tissue degeneration by preserving cells that have little regenerative potential, such as neurons and cardiomyocytes. These actions might have important consequences for the long-term survival of the organism. Accordingly, hyperactivation of pro-apoptotic factors such as p53 have been shown to lead to early aging phenotypes in mice (Tyner et al., 2002).

In a parallel manner, SirT1 also promotes survival by enhancing the cell's ability to deal with oxidative stress. It does this mainly through its interactions with the FOXO (Forkhead) transcription factors, FOXO3a and FOXO4 (Michan and Sinclair, 2007). However, unlike the previous examples, SirT1 appears to have an activating role in this instance. In the first case, SirT1 deacetylates FOXO3a and facilitates its nuclear translocation upon oxidative stress (Nemoto et al., 2004). FOXO3a, which is normally found inactive in the cytoplasm, binds to SirT1 once in the nucleus and together they upregulate the expression of repair and cell cycle checkpoint genes. This promotes cell cycle arrest and the repair of cellular damage, thereby preventing the onset of apoptosis. FOXO4 also displays a similar regulatory profile where its deacetylation by SirT1 promotes the transcription of cell cycle checkpoint and oxidative stress genes (van der Horst et al., 2004). Since oxidative damage caused by reactive oxygen species is thought to be a cause of aging, SirT1's protective role in this regard is consistent with a role in

longevity. Intriguingly, SirT1 expression has recently been found to be upregulated in response to oxidative damage, possibly as a defense mechanism (Haigis and Sinclair, 2006).

However, given the abundance of evidence implicating SirT1 as an anti-apoptotic factor, it would not be unreasonable to assume that SirT1 might also be pro-oncogenic. This has in fact been proposed: the HIC1 gene is often found hypermethylated in human tumors, which results in overexpression of SirT1 and inactivation of p53. In spite of its many pro-survival activities, SirT1 has also been shown to act as a tumor suppressor in several different tissues. This is best exemplified by the interaction of SirT1 with NF κ B (nuclear factor kappa-B); SirT1 is known to deacetylate the RelA/p65 component of NF κ B and thereby repress its transcriptional activity (Yeung et al., 2004). This in turn has been shown to sensitize human cells to apoptosis in response to TNF α (tumor necrosis factor alpha), which has been confirmed in human breast cancer cells. More recently, SirT1 has been found to also protect against a model of colon cancer *in vivo* by deacetylating β -catenin and repressing its transactivation potential (Firestein et al., 2008). Since β -catenin is known to play an important oncogenic role in various other human malignancies, including skin and prostate cancer, SirT1 would be predicted to have a tumor suppressive role in these tissues as well.

The role of SirT1 in carcinogenesis is therefore more complicated than originally thought. However, given that CR is generally known to be protective against cancer, it is possible that SirT1 acts in a more general manner to promote stress resistance and cell survival, while acting in a tissue specific manner to protect against transformation. And at least in the aforementioned examples, these tissue specific effects appear to override the more general anti-apoptotic ones. Consistent with this, a new study shows that overexpression of SirT1 in the brain

protects against irradiation-induced cancer in mice (Oberdoerffer et al., 2008). However, suffice it to say that the exact role of SirT1 in carcinogenesis still remains highly controversial.

Regulation of metabolism

Metabolism is another pathway over which SirT1 exerts a dramatic influence. For instance, SirT1 has been shown to promote fat mobilization by interacting with and repressing PPAR γ (Peroxisome proliferator-activated receptor gamma), the master fat transcription factor (Picard et al., 2004). Interestingly, this interaction only occurs when mice are fasted, suggesting SirT1 might regulate the physiological adaptation to food insufficiency during CR. In line with this finding, heterozygous SirT1 knockout mice (+/-) show reduced lipolysis upon fasting (Picard et al., 2004). Intriguingly, since a reduction in fat is sufficient to increase lifespan (albeit not to the same extent as CR), this finding may provide a direct link between SirT1 and longevity (Blüher et al., 2003).

In addition, SirT1 is also known to exert many of its metabolic effects through the deacetylation and activation of the PPAR γ co-activator, PGC-1 α , a master regulator of mitochondrial biogenesis (Rodgers et al., 2005). This activation results in different outputs depending on the tissue: in the liver it represses glycolysis and upregulates gluconeogenesis; in muscle it increases mitochondrial biogenesis and leads to greater metabolic activity and improved endurance; in brown fat, PGC-1 α activation leads to enhanced mitochondrial activity and improved thermogenesis (Michan and Sinclair, 2007). These disparate effects lead to an overall increase in metabolic rate, resulting in increased glucose metabolism and improve insulin sensitivity - consistent with what is observed with CR. The positive role of SirT1 in metabolism has been validated *in vivo*: both SirT1 transgenic mice, as well as mice treated with SirT1 agonists such as resveratrol, show lower blood glucose levels and increased insulin sensitivity

(Lagouge et al., 2006; Baur et al., 2006). Furthermore, resveratrol has recently been shown to protect against the metabolic disorders associated with a high fat diet in mice. There is even evidence that these effects might extend to humans – polymorphisms in the SirT1 gene were recently shown to associate with overall energy expenditure in a Finnish population (Lagouge et al., 2006).

In addition to its indirect effects on insulin sensitivity, SirT1 also directly influences the secretion of insulin from pancreatic β -cells (Bordone et al., 2006; Moynihan et al., 2005). Animal studies show that SirT1 whole-body knockout mice have reduced insulin secretion, while β -cell specific SirT1 transgenic mice have increased insulin output. It appears that SirT1 mediates these effects by repressing the expression of UCP2 (uncoupling protein 2). This in turn leads to enhanced coupling of mitochondrial respiration and ATP synthesis, resulting in increased insulin secretion. Consistent with this model, SirT1 knockout mice fail to show elevated ATP production in response to glucose, while β -cell specific transgenic mice show elevated ATP levels. Interestingly, SirT1 repression of UCP2 actually weakens upon food withdrawal, resulting in lower insulin secretion (Bordone et al., 2007). In hindsight, these findings should not be too surprising; β -cells have previously been reported to sense glucose by the conversion of NAD to NADH (Koubova and Guarente, 2003).

One of the last ways SirT1 exerts its influence on metabolism is through its interaction with the Forkhead family member, FOXO1. In pancreatic β -cells, SirT1 deacetylates FOXO1 and thereby induces expression of the β -cell transcription factors, NeuroD (neurogenic differentiation) and MafA (musculoaponeurotic fibrosarcoma homolog A) (Motta et al., 2004; Michan and Sinclair, 2007). These in turn increase the stress resistance of the islet cells and help preserve insulin secretion. In the liver, on the other hand, SirT1's activation of FOXO1 (and

PGC-1 α) promotes the transcription of hepatic gluconeogenic genes during times of fasting (Haigis and Guarente, 2006). SirT1 therefore appears as a major regulator of the hepatic response to changing nutrient conditions, though recent evidence suggests that SirT1 activity might actually decline in the liver during CR, suggesting further studies are needed (Chen et al., 2008).

The above studies show a remarkable overlap in the metabolic processes regulated by SirT1 and those of CR. This association has been further strengthened by recent evidence directly linking SirT1 with CR. For instance, endothelial nitric oxide synthase (eNOS) has been shown to mediate the increases in respiration and mitochondrial biogenesis associated with CR, possibly through induction of SirT1 (Nisoli et al., 2005). Consistent with this hypothesis, eNOS knockout mice show neither activation of SirT1, nor increased mitochondrial output upon CR. SirT1 knockout mice also fail to respond to several physiological aspects of CR, including increased activity and upregulation of metabolism (Cohen et al., 2004; Chen et al., 2005; Boily et al., 2008). Conversely, mice treated with resveratrol show a transcriptional profile that closely resembles that of CR. Finally, SirT1 activity is thought to be induced in certain tissues during CR due to increased NAD/NADH levels, as well as expression of SirT1 (Cohen et al., 2004; Nisoli et al., 2005; Chen et al., 2008). However, the story might be more complicated than originally thought – in some tissues, such as the liver, pancreas and certain regions of the brain, SirT1 expression and NAD/NADH levels appear to decrease upon CR (Chen et al., 2008). It is therefore likely that the disparate effects of SirT1 in different tissues may require coordination of SirT1 activity that goes beyond simple activation.

Effects on cellular differentiation

Another aspect of mammalian physiology that SirT1 regulates is cellular differentiation. As mentioned before, SirT1 represses the master fat transcription factor, PPAR γ , and thereby

inhibits adipogenesis (Picard et al., 2004). In a mechanism that is not clearly understood, SirT1 interacts with PPAR γ and represses transcriptional activation of its downstream targets, including many of the major adipocyte genes. As a result, overexpression of SirT1 decreases differentiation of the 3T3-L1 pre-adipocyte cell line, while RNAi (RNA interference) of SirT1 increases it. In addition, the small molecule SirT1 activator, resveratrol, also represses fat differentiation of 3T3-L1s in a dose dependent manner. These effects have been validated *in vivo* as well – mice on a high fat diet treated with resveratrol gain less weight and have reduced fat deposits. Further, our lab has recently confirmed that fat specific SirT1 knockout mice show increased adiposity, confirming SirT1 represses fat differentiation *in vivo* (Angeliki Chalkiadaki, personal communication).

Besides fat, SirT1 has also been shown to regulate the differentiation of myocytes. Muscle is unique in that it is a highly metabolic tissue in which NAD/NADH levels change dramatically with activity and differentiation. Fulco and colleagues demonstrated that these changes were important for differentiation: reductions in NAD/NADH promotes muscle differentiation by repressing SirT1 (Fulco et al., 2003). Further, this group demonstrated that SirT1 interacts with MyoD, the master muscle transcription factor, and represses its transcriptional activity. This repression is physiologically relevant – RNAi of SirT1 enhanced differentiation of the C2C12 myoblast cell line, while overexpression repressed it. Consistent with this, small molecule inhibitors of SirT1 activated transcription of MyoD targets. However, whether SirT1 is a regulator of muscle differentiation *in vivo* has yet to be validated. It is worth noting that neither SirT1 transgenic mice, nor mice treated with resveratrol, have been reported to have reduced muscle mass.

Unlike its role in fat and muscle, SirT1 appears to promote the differentiation of chondrocytes and keratinocytes (Dvir-Ginzberg et al., 2008; Blander et al., 2009). In both cases, SirT1 overexpression (or resveratrol treatment) increases differentiation *in vitro*, whereas RNAi of SirT1 attenuates it. Although the mechanism is currently unknown in keratinocytes, in chondrocytes it appears that SirT1 deacetylates Sox9, the master cartilage transcription factor, and thereby activates its transactivation potential. Interestingly, Sox9 also appears to target SirT1 to the promoter of its target genes, where SirT1 recruits transcriptional co-activators such as the histone acetyltransferases, p300 and GCN5 (Dvir-Ginzberg et al., 2008). It certainly is odd for a histone deacetylase such as SirT1 to *activate* gene expression, let alone activate it through recruitment of histone acetyltransferases. However, it is worth noting that SirT1 has been shown to interact with p300, albeit in a negative manner (Motta et al., 2004).

How these disparate effects of SirT1 on cellular differentiation relate to CR is still an open question. However, it is worth noting that fat, muscle, cartilage and bone are all derived from a common mesenchymal stem cell population in the bone marrow (which will be discussed later). It is possible that during times of food scarcity, an animal might repress differentiation of fat and muscle in order to preserve limited resources. In this regard, progenitor cells would accumulate which could replenish lost muscle or fat once food becomes readily available again. Another possible scenario is that by repressing the muscle and fat pathways, SirT1 might actually indirectly stimulate the formation of connective tissue such as cartilage and bone. These tissues might actually be in greater demand during CR since animals are known to dramatically increase their physical activity, possibly as a foraging response.

Sirt1 animal models

Two independent lines of SirT1 knockout mice have been made – one that has the catalytic domain of SirT1 deleted and one that results in complete loss of the SirT1 protein (Cheng et al., 2003; McBurney et al., 2003). The two lines are nearly identical and demonstrate that deletion of SirT1 is lethal in an inbred background, though viable mice can be obtained in an outbred background (at sub-Mendelian frequencies). This reduced viability is attributed to at least two factors: 1) the immediate postnatal death of abnormal fetuses; and 2) failure of pups to thrive during postnatal development. In fact, most knockout pups die during the first few months after birth, though ones that survive can live well into adulthood (Boily et al., 2008).

Intriguingly, a cause of prenatal death has been attributed to exencephaly, or the failure of the skull to fully enclose the brain. This suggests a role of SirT1 in bone modeling. Consistent with this idea, SirT1 knockout mice also show defects in the closure of craniofacial sutures, as well as delays in mineralization of the skull, vertebrae and digits (McBurney et al., 2003; Cheng et al., 2003). However, the role of SirT1 in the maintenance of adult bone mass has yet to be explored.

SirT1 whole-body knockouts display a number of other gross developmental defects. For instance, knockout mice have eyes which never fully open (and which often becomes infected later in life). They also are significantly smaller and weigh less than their wildtype counterparts (usually by 30-50%); and both sexes are sterile, which in males is due to abnormal sperm development and in females to hormone deficiencies (McBurney et al., 2003). Surprisingly, the knockout mice eat more food per body weight and appear to be hypermetabolic, suggesting inefficient energy metabolism (Boily et al., 2008).

Interestingly, recent studies show that SirT1 knockout mice respond to only some aspects of CR. While SirT1 knockouts show similar changes in blood glucose, triglycerides and IGF-1

levels as wildtype animals, they fail to show an increase in activity upon CR (Chen et al., 2005). Given that SirT1 knockout mice move as well or even better than wildtype mice under rotarod and treadmill tests, this observation suggests that SirT1 might regulate a possible foraging response to CR. Another study found that SirT1 knockout mice also fail to show many of the metabolic changes associated with CR, including an increase in respiration (Boily et al., 2008). Further, the authors show that knockout mice have shortened lifespans and do not live longer when placed on CR. Unfortunately, the pleiotropic effects associated with these mice have made any clear conclusions difficult to make.

In addition to knockout models, three different SirT1 transgenic models have also recently been developed (Bordone et al., 2007; Banks et al., 2008; Pfluger et al., 2008). The first used a construct that fused SirT1 to the tail end of the endogenous Actin gene, leading to a hybrid Actin-SirT1 transcript under control of the Actin promoter. Translation of SirT1 was made possible by the inclusion of an internal IRES (Internal Ribosomal Entry Site) after the end of the Actin gene. The other two models used bacterial artificial chromosomes (BACs) that allowed overexpression of SirT1 under its own endogenous promoter. In all three cases, transgenic mice display phenotypes that closely resemble aspects of CR. Notably, mice overexpressing SirT1 are insulin sensitive and glucose tolerant; they are protected against the metabolic defects associated with diet induced obesity; and show a healthy hormone profile that is associated with longevity. In addition, the pro-inflammatory cytokines Tumor Necrosis Alpha (TNF α) and Interleukin-6 (IL-6) appear to be lower in SirT1 transgenics, resulting in protection against inflammatory diseases. While lifespan studies have not been published from the latter two transgenic models, our lab has found that the Actin-SirT1 transgenics did not live longer than wildtype controls (Bordone et al., 2007).

The other mammalian Sirtuins: SirT 2 - 7

Besides SirT1, recent studies have begun to unravel the functions of the other six mammalian Sirtuins. SirT2, a predominantly cytoplasmic Sirtuin, has been shown to deacetylate tubulin, though the physiological consequences of this interaction are not clear. SirT2 might also be important in regulation of the cell cycle: it has been shown to associate with chromatin during the G₂/M transition (when the nuclear membrane is broken down) and deacetylate histone H4, thereby facilitating the formation of heterochromatin (Michan and Sinclair, 2007). Accordingly, overexpression of SirT2 blocks chromosomal condensation and delays mitosis, whereas its deletion results in hyperacetylation of histone H4 and delays entry into S-phase. SirT2 might also have a tumor suppressive role: it is commonly found deleted in gliomas and its overexpression inhibits *in vitro* colony formation of glioma cell lines (Haigis and Guarente, 2006). These findings suggest that SirT2 might therefore be a novel mitotic checkpoint protein with a possible role in cancer biology.

Unsurprisingly, the mitochondrial Sirtuins, SirT3, SirT4, and SirT5, all have important metabolic functions (Guarente and Picard, 2005). SirT3 is localized to the mitochondrial matrix where it appears to regulate mitochondrial function. Specifically, overexpression of SirT3 increases respiration and decreases reactive oxygen species. However, in brown fat SirT3 has actually been shown to uncouple ATP production from respiration. This uncoupling occurs through induction of Uncoupling Protein 1 (UCP1), which helps promote adaptive thermogenesis. However, the physiological consequences of this regulation are not clear: SirT3 knockout mice do not show a defect in adaptive thermogenesis (Danica Chen, personal communication).

More recently, SirT3 has also been shown to deacetylate and thereby activate acetyl-CoA synthetase (AceCS), the enzyme responsible for production of acetyl-CoA (a TCA cycle intermediate that is the building block for cholesterol and fatty acids) (Haigis and Guarente, 2006). In this regard, SirT3 might potentially regulate a crucial aspect of metabolism: the shuttling of carbon for different metabolic processes. The physiological consequences of this regulation have also yet to be explored.

SirT4 regulates an important, albeit different, aspect of metabolism – the secretion of insulin by pancreatic β -cells. Unlike the other mitochondrial Sirtuins, SirT4 does not appear to have deacetylase activity, instead catalyzing an ADP-ribosyltransfer reaction. An important target of this reaction is glutamate dehydrogenase (GDH), an enzyme that catalyzes the conversion of glutamate into α -ketoglutarate, another TCA cycle intermediate. In the pancreas, GDH is a key regulator of amino acid-stimulated insulin secretion and by repressing GDH, SirT4 represses insulin secretion (Haigis et al., 2006). Consistent with this model, SirT4 knockout mice show both higher GDH activity and insulin secretion. Similar to SirT1, SirT4 expression decreases in the pancreas during CR, resulting in induction of GDH and increased amino acid-stimulated insulin secretion. As a result, it is thought that SirT4 mediates a shift in which amino acids gain more importance in regulating insulin secretion during CR (Haigis and Guarente, 2006). Notably, the downregulation of SirT4 in the pancreas upon CR provides another example in which CR appears to have either an activating or repressive role depending on the Sirtuin and tissue.

The final mitochondrial Sirtuin, SirT5, is the least characterized member of the family. Our lab has recently demonstrated that SirT5 localizes to the inner mitochondrial matrix where it deacetylates and activates carbamoyl-phosphate synthetase 1 (CPS1), the rate-limiting enzyme in

the first step of the hepatic urea cycle (Takashi Nakagawa, personal communication). This activation is important for ammonia detoxification during times of food scarcity, where breakdown of proteins for energy use results in the accumulation ammonia. Consistent with this, SirT5 knockout mice have higher circulating ammonia levels during fasting. Interestingly, SirT5 activity increases during CR due to increased mitochondrial NAD/NADH levels, resulting in stimulation of CPS1 activity and the resulting detoxification of ammonia.

Finally, the nuclear Sirtuins, SirT6 and SirT7, mediate processes that are important for DNA repair and rDNA transcription, respectively (Michan and Sinclair, 2008). SirT6 appears to have both deacetylase and ADP ribosyltransferase activity, whereas SirT7 only shows deacetylase activity. SirT6 knockout mice display many severe phenotypes that have been suggested to be ‘a premature aging’ syndrome. For example, knockout mice are smaller than wildtypes, and have reduced bone mineral density and subcutaneous fat; and display signs of kyphosis, lymphocyte apoptosis, colitis and all die within 4 weeks of age (Mostoslavsky et al., 2006). Many of these phenotypes mirror those seen knockouts of other DNA repair enzymes, and accordingly SirT6 is proposed to be an important mediator of base excision repair (BER). Consistent with this, SirT6 knockout MEFs showing impaired proliferation, great sensitivity to DNA damaging agents, and genomic instability – many of which can be rescued via overexpression of the DNA polymerase involved in BER, Pol β (Mostoslavsky et al., 2006). Though, exactly how SirT6 promotes BER is currently unknown.

More recently, SirT6 has been shown to have histone deacetylase activity, with preference for Histone H3. This silencing is associated with telomeric silencing and repression of NF κ B targets, much like that seen for SirT1.

SirT7, the only Sirtuin localized to the nucleolus, appears to activate rDNA transcription via its interaction with RNA polymerase I (Pol I) (Ford et al., 2006). Though the mechanism remains unclear, overexpression of SirT7 increases Pol I mediated transcription of rDNA, while RNAi inhibits it. This activation appears essential for cell proliferation and viability in human cells since knockdown of SirT7 results in cell death. However, the physiological consequences of this are unclear given that SirT7 knockout mice are viable and grossly normal. In contrast, recent studies have also proposed a tumor suppressive role for SirT7: overexpression of SirT7 inhibits cell proliferation, and low SirT7 expression correlates with the tumorigenic potential of several murine cell lines (Haigis and Sinclair, 2006).

Recent *in vivo* studies support a role for SirT7 in cell survival and stress resistance. SirT7 knockouts develop heart hypertrophy and inflammatory cardiomyopathy due to increased apoptosis (Michan and Sinclair, 2008). Much like SirT1, SirT7 appears to mediate these effects through its interaction with p53: SirT7 interacts with and deacetylates p53 *in vitro*, and knockout mice show hyperacetylation of p53. It is also interesting to note that SirT7 knockout mice show dramatically reduced lifespan, suggesting a potential role in longevity as well. Additionally, the overlapping functions of SirT7 and SirT1 in p53 regulation, as well as SirT6 and SirT1 in repression of NFκB signaling, suggest possible redundancy and/or crosstalk between different members of the Sirtuin family.

BONE DEVELOPMENT, REMODELING, AND AGING

Bone is a specialized form of connective tissue that, together with cartilage, makes up the skeletal system. Bone consists of an organic extracellular matrix composed primarily of collagen type I fibers (called osteoid), on which inorganic crystals of hydroxyapatite [$3\text{Ca}_3(\text{PO}_4)_2(\text{OH})_2$] are secreted (though other ions such magnesium, potassium and citrate can also be found). In

addition to collagen, the extracellular matrix is composed of other proteins such as Osteopontin, Bone Sialoprotein, Osteocalcin, and other glycoproteins that help promote calcification due to their high ion binding affinity. Mineralized bone serves three main functions: (1) it provides mechanical support and promotes locomotion by providing an anchor for attachment of muscle fibers; (2) it provides protection for vital organs, as well as an environment for hematopoiesis; and (3) it provides a reservoir for important inorganic ions such as calcium and phosphate. In addition to these canonical functions, recent studies suggest bone can also serve an endocrine role by secreting hormones such as Osteocalcin that coordinate with other tissues to regulate metabolism (Lee et al., 2007).

Anatomically, there are two main types of bone in the skeletal system: long bones such as the tibia and femur, and flat bones such as those found in the skull. Long bones consist of an outer ring of dense compact (or cortical) bone, as well as an inner, more hollow meshwork called trabecular or spongy bone (**Figure 5A**). It is within this inner cavity that the bone marrow is found and most of hematopoiesis occurs. Flat bones on the other hand, are composed completely of dense cortical bone and primarily function to help protect internal organs.

Bone development and remodeling

In addition to these anatomical differences, flat and long bones are also formed by two different developmental processes. Flat bones arise through intramembranous ossification, in which mesenchymal stem cells (MSCs) directly differentiate into osteoblasts. Long bones, on the other hand, undergo endochondral ossification in which MSCs first differentiate into chondrocytes which lay a cartilage anlagen of the future skeleton. This cartilage model is later invaded by osteoblasts and replaced with mineralized bone. These two developmental processes

encompass bone modeling, which is the formation of the skeletal system during development. This is in contrast to bone remodeling, which is the maintenance of bone mass during adulthood.

Both bone modeling and remodeling require the concerted action of the two principle bone cells: the bone forming osteoblasts, and the bone resorbing osteoclasts. Misregulation of these cells is implicated in diseases of the skeleton: during development it results in skeletal defects, whereas during adulthood it leads to osteoporosis. Further, the lifelong process of bone remodeling underscores the fact that, in spite of its appearance, bone is actually a dynamic tissue that is continuously turned over – much in the way that Massachusetts Avenue is continuously dug up and repaved every few years. The importance of this remodeling process is pointedly demonstrated by the counterintuitive example of osteopetrosis – a condition where insufficient osteoclast activity leads to increased fracture risk in spite of elevated bone mass. Therefore, healthy bones require both sufficient bone mass and the continual remodeling of existing bone. This process inevitably breaks down during aging, resulting in osteoporosis (**Figure 5B**).

Osteoporosis, a disease of uncoupled remodeling

Osteoporosis, at its most fundamental level, results from uncoupled bone remodeling. This can result from either too little bone being made or too much being broken down. To distinguish between these two causes, the disease has been divided into two categories: Type I (post-menopausal) osteoporosis occurs in women and is characterized by hyperactivity of osteoclasts as a result of diminished estrogen levels (a natural inhibitor of osteoclasts); while Type II (age-related) osteoporosis is later onset, occurs in both sexes and is associated with decreased osteoblastogenesis.

Clinically, bone mass is measured by X-ray scans measuring bone mineral density (BMD), with osteoporosis defined as a BMD score 2.5 standard deviations below the average

peak bone mass for each particular sex. However, it is noteworthy that even smaller amounts of bone loss can have dramatic clinical consequences: it is estimated that for every 10% of bone that is lost, the risk of fracture doubles (Rodan and Martin, 2000). Moreover, according to the World Health Organization, nearly a fifth of all of post-menopausal women are osteoporotic, with an even greater number significantly at risk for developing the disease. These statistics have significant clinical outcomes: there are approximately 1.3 million fractures in the United States annually, with osteoporotic fractures (particularly those of the hip), resulting in both higher morbidity and dramatically increased healthcare costs. And as many of us with ailing grandparents can attest, a fracture at old age not only increases chance of death, but also dramatically reduces the quality of life.

Accordingly, osteoporosis has become a significant public health issue in recent years. This has arisen partly because there are currently no effective treatments for age-related osteoporosis. Most currently available drugs (such as the bisphosphonates) target osteoclasts and therefore are only useful against post-menopausal osteoporosis. These drugs work in several ways: by either coating the bone surface with a resorption-resistant layer; by inhibiting the osteoclast enzymes needed for resorption (i.e. Cathepsin-K inhibitors); or by stimulating apoptosis in osteoclasts (i.e. estrogen). Although these drugs have been tremendously useful in reducing the incidence of fractures in high-risk women, there is growing concern about their long term use (Boyle et al., 2003). This is because these drugs also effectively shut down bone remodeling, which is essential for the maintenance of bone strength. Consistent with this, women on bisphosphonates show an increased rate of osteonecrosis (bone death) due to impaired repair of damaged bone (Silverman and Landesberg, 2009). Of course, an even bigger concern is that the drugs are not suitable for the other half of the population (i.e. men), nor for treating the later

onset age-related osteoporosis. With populations in developed countries living ever longer, osteoporosis is thus predicted to become an even larger health concern. This in turn has made the discovery of anabolic drugs that promote bone growth the holy grail of osteoporosis research.

Osteoblasts, the bone forming cells of the skeleton

Osteoblasts are the cells responsible for creating bone. They accomplish this in several ways: first, they actively secrete vast amounts of extracellular proteins such as Collagen type I and numerous glycoproteins. This provides a matrix, referred to as osteoid, which helps promote calcification; largely owing to the high ion binding affinity of the secreted glycoproteins (Harada and Rodan, 2003). Osteoblasts also express high levels of Alkaline Phosphatase-2 (ALP2) on their cell surface which helps facilitate mineralization by hydrolyzing pyrophosphate ($P_2O_7^{-4}$), an anti-mineralizing chelating agent, to free phosphate ($2HP_2O_4^{-2}$). This not only removes a barrier to mineralization, but also dramatically increases the local concentration of free phosphate at the osteoblast surface, thus facilitating hydroxyapatite formation. Given its structure, bone is often compared to reinforced concrete – with collagen acting as the rebars and the minerals acting as the concrete. Working together, the mineral matrix and the underlying organic matrix provide both strength and flexibility, resulting in a superbly hard yet resilient structure.

Differentiation process

Osteoblasts are derived from mesenchymal stem cells (MSCs) in the bone marrow that also give rise to adipocytes, myocytes and chondroblasts (**Figure 5C**). Differentiation of an osteoblast involves two main steps: first, commitment of the MSC to the osteoblast lineage, followed by terminal differentiation to an osteocyte (Harada and Rodan, 2003). Accordingly, the differentiation of an osteoblast can be divided into several distinct phases, each with unique characteristics: osteoprogenitor cells actively divide and thereby maintain osteoblast populations;

pre-osteoblasts have exited the cell cycle and have initiated the early steps of differentiation; osteoblasts actively synthesize osteoid and mineralized matrix; and osteocytes have become fully embedded within the mineralized matrix. Further, cells at these different stages can be distinguished based on the expression of osteoblast markers. For example, Alkaline Phosphate and Collagen type I are early markers of differentiation; whereas Osteocalcin, Bone Sialoprotein, Osteopontin and mineralization are associated with more mature osteoblasts.

Runx2 and Osterix: the major osteoblast transcription factors

The exact molecular processes mediating osteoblast differentiation remained largely a mystery until relatively recently. In 1997, multiple reports outlined the discovery of the master osteoblast transcription factor, Runx2 (Runt-related transcription factor 2) (Ducy et al., 1997; Komori et al., 1993; Otto et al., 1993). The evidence was compelling: Runx2 knockout mice lacked a mineralized skeleton due to a complete lack of osteoblast differentiation. Rather, they contained a cartilage framework of the skeleton. Interestingly, in addition to a lack of osteoblasts, Runx2 knockouts also lacked hypertrophic chondrocytes, suggesting a role for Runx2 in the terminal differentiation of cartilage as well. Further, overexpression of Runx2 was sufficient for the induction of the osteoblast program, even in non-osteoblastic cells. And finally, hypomorphic mutations in Runx2 were found to be the hereditary cause of the human skeletal disorder, cleidocranial dysplasia (CCD). Today, Runx2 is a well established master regulator of osteoblast differentiation.

Runx2 is a member of the Runx family of transcription factors, which includes Runx1-3. These all share sequence homology with the *Drosophila* Runt transcription factor and contain a conserved 128 amino acid core Runt homology domain which mediates DNA binding. Further, all three Runx family members have been shown to play important roles in cellular

differentiation: Runx1 is required for haematopoiesis; Runx3 plays an important role in development of the nervous system; and Runx2 controls osteoblastogenesis (Harada and Rodan, 2003).

Runx2 mediates its effects by binding to osteoblast specific cis-acting elements (OSE2) in the promoter of target genes. Consequently, Runx2 has been shown to mediate the expression of nearly all of the major osteoblast genes, including Osteocalcin, Osteopontin, and Bone Sialoprotein. However, recent data shows that non-Runx2 mediated activation of these genes is also possible, suggesting the existence of other important transcription factors. This hypothesis was confirmed with the cloning of a second essential osteoblast transcription factors, Osterix (Osx/Sp7) (Nakashima et al., 1997). Indeed, Osx knockout mice also show the complete lack of a mineralized skeleton. However, unlike Runx2 nulls, these mice have normal hypertrophic chondrocytes, suggesting a more specific role of Osx in osteoblast differentiation. Consistent with this, overexpression of Osx is sufficient to promote various aspects of osteoblast differentiation.

Since Osx is not expressed in Runx2 knockouts, while Runx2 is expressed in Osx knockouts, it is commonly thought that Osx is genetically downstream of Runx2 (Ducy et al., 1997; Nakashima et al., 1997). Consistent with this, OSE2 elements have been described in the promoter region of Osx, and overexpression of Runx2 has been shown to activate transcription of Osx. While Runx2 is sufficient for Osx expression, it appears that Osx expression can also be induced by additional factors (discussed further below). The present data suggests that Runx2 mediates the early steps of differentiation (including induction of Osx), while Osx is required for the later events, including mineralization.

Osteoblast commitment

While Runx2 and Osx work together to promote terminal differentiation of osteoblasts, other signaling pathways mediate the earlier events in osteoblastogenesis. For example, there appears to be a special relationship between osteoblasts and adipocytes during the commitment of the early mesenchymal stem cell (MSC) progenitor: the two lineages appear mutually exclusive and commitment down one lineage appears to inhibit the other. Moreover, as mammals age, osteoblast numbers decrease while adipocyte numbers increase, leading to an accumulation of fat in tissues that normally do not store fat, such as the bone marrow. Intriguingly, pharmacological activation of PPAR γ , the master fat transcription factor, with the antidiabetic thiazolidinediones also leads to a similar phenotype where the bone marrow becomes 'fatty' and osteoblast numbers decline (Rzonca et al., 2004). Inversely, PPAR γ +/- mice are not only resistant to obesity and diabetes but also have increased osteoblastogenesis and bone mass (Akune et al., 2004). These data suggest that misregulation in the proper balance between adipogenesis and osteoblastogenesis during the aging process (possibly through hyperactivation of PPAR γ) might be a cause of age-related osteoporosis.

It also appears that signals from the local microenvironment likely play an important role in the commitment of MSCs (Harada and Rodan, 2003). Consistent with this, MSCs have recently been isolated from other mesenchymal tissues such as fat and muscle, suggesting MSC populations exist outside of the bone marrow. Since MSCs in these different tissues would be exposed to different microenvironments, it is thought that cytokines and other signaling molecules play key roles in initiating tissue specific transcription programs.

In bone, the bone morphogenetic proteins (BMPs) represent such factors. BMP proteins, members of the TGF- β superfamily, play key roles in skeletal development and osteoblast differentiation (Marie, 2008). For instance, BMP2 leads to ectopic bone growth when injected

into the fat pads of mice, and dramatically increases differentiation of osteoblast cells *in vitro* (Chen et al., 2004). BMP2 does this, in part, by upregulating the transcription and activity of both Runx2 and Osx, largely through the SMAD signaling pathway. Moreover, certain SMADs have also been shown to directly bind Runx2 and activate transcription of its downstream genes. Recent studies show BMP2 also induces TAZ (Transcriptional co-activator with PDZ-binding motif), which simultaneously activates Runx2 and represses PPAR γ (Hong et al., 2005). This in fact explains how BMP2 both promotes osteoblast, and represses adipocyte differentiation in MSCs. Finally, BMP signaling also activates Runx2 transcriptional activity through the mitogen activated kinase (MAPK) signaling pathway (Celil and Campbell, 2005).

Early steps of differentiation

Recent evidence has indicated that other factors are important for the early steps of osteoblast differentiation. The most important of these are the homeobox proteins including Msx2 (Msh homeobox homolog) and the distal-less homeobox (Dlx) family of transcription factors. Msx2, Dlx3 and Dlx5 are expressed and upregulated at the early stages of osteoblast differentiation in response to BMP2 (Matsubara et al., 2008; Hassan et al., 2004). Consistent with a role in bone development, mutations in these genes are associated with skeletal defects.

Clues to the function of Msx2 came when inactivating mutations were found to cause both reduced Runx2 expression and delays in skull ossification. Conversely, overexpression of Msx2 has been shown to enhance osteoblast differentiation through upregulation of Runx2 transcription (Matsubara et al., 2008). And in an interesting twist, Msx2 has also been shown to upregulate Osx expression, even in the absence of Runx2 – possibly explaining the previously described Runx2 independent expression of osteoblast markers.

In a similar manner, Dlx3 and Dlx5 also appear to be important for BMP2 induction of Runx2 and Osx. Interestingly, Dlx5 appears essential for the BMP2 induction of Osx - RNAi of Dlx5 completely abrogates this effect (Hassan et al., 2004; Harada and Rodan, 2003). Although much of this work has been done *in vitro*, the present data suggests that Msx2, Dlx3 and Dlx5 are important factors for the initiation of the osteoblast transcription program, likely through induction of Runx2 and Osx.

Osteoclasts, the bone resorbing cells of the skeleton

Unlike osteoblasts, osteoclasts are haematopoietic cells derived from a monocytic lineage (which also gives rise to macrophages) (**Figure 5D**). Osteoclasts are polykaryons formed from the fusion of monocyte precursors and are the cells responsible for breaking down (resorbing) old bone. Since any misregulation in their activity could potentially lead to either too little bone (osteoporosis) or too much bone (osteopetrosis), their activity is tightly regulated by their osteoblast counterparts.

Osteoblasts regulate the differentiation of osteoclasts through three main factors: MCSF (macrophage colony stimulating factor), RankL (receptor activator of NF κ B ligand), and OPG (osteoprotegerin). The first two factors, MCSF and RankL, promote and together are sufficient for osteoclast differentiation, while OPG inhibits the process by sequestering RankL (Boyle et al., 2003).

Osteoblasts actively secrete MCSF, which upon binding to the c-Fms receptor, promotes the survival and proliferation of the monocyte precursor (Teitelbaum, 2000). RankL, on the other hand, is a transmembrane ligand on the osteoblast surface that binds directly to the Rank receptor on the osteoclast surface. RankL is responsible for initiating the early events required for osteoclast differentiation, including activation of NF κ B. Both MCSF and RankL are absolutely

necessary for osteoclast differentiation - mice with inactivating mutations in either factor do not produce osteoclasts and become osteopetrotic (Boyle et al., 2003). Conversely, osteoblasts can also attenuate osteoclast differentiation by secreting OPG, a 'decoy receptor' which competes with the osteoclast Rank receptor for binding to RankL. As a result, OPG is a potent inhibitor of osteoclast differentiation - mice lacking OPG develop osteoporosis due to increased osteoclastogenesis.

Once differentiated, the osteoclast undergoes internal structural changes that prepare it to resorb bone, giving it the classic 'ruffled membrane' appearance. It first rearranges its Actin cytoskeleton and forms a tight junction with the bone surface, thus creating a sealed compartment (Teitelbaum, 2000). It then acidifies this compartment by pumping hydrogen ions (via the ATP6i complex) and secreting TRAP (tartate-resistant acid phosphatase), which efficiently demineralizes the bone. This is followed by secretion of the lysosomal protease, Cathepsin K, which digests the underlying organic matrix. The resulting degradation products are then processed by the osteoclast and released into circulation. The osteoclast then undergoes apoptosis, with nearby osteoblasts beginning the work of replacing the resorbed bone.

Osteoclast differentiation

The differentiation process of osteoclasts is comparatively less complex than that of osteoblasts. Unlike the mesenchymal stem cell progenitor, the monocyte precursor is only bipotential and differentiation down one lineage does not appear to inhibit the other. In fact, MCSF is required for both macrophage *and* osteoclast differentiation, and in the absence of RankL, will differentiate the monocyte precursor to a mature macrophage. Consistent with this, deletion of MCSF in mice leads to both a lack in osteoclasts and macrophages (Boyle et al., 2003). Therefore macrophages and osteoclasts are actually very much alike, with osteoclasts

often portrayed as specialized macrophages. Further, since MCSF is required for both macrophage and osteoclast differentiation, it appears that RankL is the critical osteoclast differentiation factor (Teitelbaum, 2000).

Both MCSF and RankL mediate their effects through receptor mediated signaling cascades. MCSF binds to the Fms receptor, a tyrosine kinase, which transmits its signal via Src and PI3-Kinase (Boyle et al., 2003). In addition, MCSF also activates MITF (microphthalmia-associated transcription factor) which upregulates expression of anti-apoptotic proteins such as Bcl-2. Moreover, MCSF also primes the cell for differentiation towards osteoclasts by inducing expression of the Rank receptor. This has been confirmed experimentally – cells treated only with RankL fail to differentiate towards osteoclasts. There also appears to be growing evidence that MCSF also mediates the later steps of osteoclast activity, particularly events associated with motility and cytoskeletal rearrangement (Teitelbaum, 2000).

NFκB, a critical mediator of osteoclastogenesis

Unlike the Fms receptor, the Rank receptor lacks any intrinsic enzymatic activity, instead mediating its signal via recruitment of the TRAF (TNF receptor-associated factor) family of proteins. Although the exact mechanism is not clearly understood, RankL binding to the Rank receptor induces receptor trimerization and recruitment of adaptor molecules, including TRAF6. This complex in turn activates the NFκB signaling pathway, as well other pathways including the mitogen activated kinases (MAPK) and activator protein-1 (AP1). However, genetic evidence predominantly supports an essential role for NFκB in osteoclast differentiation (Asagiri and Takayanagi, 2007).

As evidenced by its name, one of the earliest molecular events induced by Rank (Receptor Activator of NFκB) is activation of NFκB. NFκB is a family of dimeric transcription

factors that bind to a common DNA sequence called the κ B site. In mammals, there are five NF κ B protein: Rel (cRel), RelA (p65), RelB, NF κ B1 (p50), and NF κ B2 (p52). The Rel family members contain transcriptional activation domains, whereas p50 and p52 do not (and thus require heterodimerization with Rel proteins for transcription activation). In non-stimulated cells, NF κ B family members reside in the cytoplasm but rapidly translocate to the nucleus upon stimulation, where they promote transcription of target genes. NF κ B agonists such as RankL activate the Inhibitor of the κ B Signaling Pathway Kinase (IKK), which phosphorylates and promotes the ubiquitin-dependent degradation of I κ Bs (the proteins responsible for sequestering the NF κ Bs in the cytoplasm). Upon degradation of I κ B, NF κ B enters the nucleus and activates transcription of target genes. Additionally, an alternative activation pathway has been shown for the p52:RelB dimer, which involves processing of the cytoplasmic p100:RelB precursor to the active p52 form. This pathway is essential for osteoclastogenesis: inactivating mutations in p50 and p52, or overexpression of I κ B α , both result in a block in osteoclastogenesis (Asagiri and Takayanagi, 2007).

NFATc1, a master osteoclast transcription factor

Once in the nucleus, a critical target of NF κ B is NFATc1 (Nuclear Factor of Activated T-cells). NF κ B dramatically activates expression of NFATc1 by binding directly to κ B sites in its promoter (Takayanagi, 2007). This induction appears essential for osteoclastogenesis: NF κ B inhibitors that prevent activation of NFATc1 completely block osteoclast differentiation, as do mutations in NFATc1 (Boyle et al., 2003; Takayanagi, 2007). Conversely, NFATc1 overexpression can drive osteoclastogenesis even in the absence of RankL (Asagiri and Takayanagi, 2007). Since overexpression of NF κ B is sufficient for activation of NFATc1, NF κ B

is thought to act as a critical intermediary between RankL and NFATc1 in osteoclastogenesis (Asagiri and Takayanagi, 2007).

Once induced, NFATc1 directly mediates the expression of many of the major osteoclast genes, including TRAP, Calcitonin Receptor, Cathepsin K, and β 3 Integrin genes (Takayanagi et al., 2002; Ishida et al., 2002). Further, NFATc1 also promotes its own expression, thus establishing a positive feedback loop that ensures the stable expression of the osteoclast program. As a result, NFATc1 is regarded as a master osteoclast transcription factor, though the exact molecular details are still being worked out.

SUMMARY

As we have discussed, aging appears to be an inevitable aspect of life, though the rate of aging can clearly be regulated. Given the evidence for the role of CR, and its downstream effector Sir2 in promoting longevity, here we have asked two questions: (1) Does mammalian SirT1 play any role in regulating bone mass; (2) Does CR offer protection against osteoporosis. To answer the first question, we have analyzed different knockout models of SirT1: Chapter 2 presents data on SirT1 whole-body knockouts, while Chapters 3 and 4 explore the role of SirT1 in osteoblasts and osteoclasts, respectively. Finally, in Chapter 5 we show that CR in fact does lead to gains in bone mass, possibly countering the effects of osteoporosis. Importantly, we find that these effects are abrogated in whole-body SirT1 knockout mice, suggesting SirT1 acts downstream of CR in bone. Our studies therefore provide the first evidence linking SirT1 to bone remodeling, metabolism, and possibly protection against osteoporosis. Given the current lack of drugs for age-related osteoporosis, we propose that activators of SirT1 (and other potential CR mimetics) represent strong candidates as new therapies for the disease.

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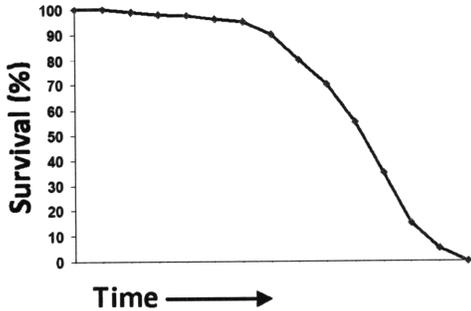
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Figure 1. Mortality rates associated with aging.

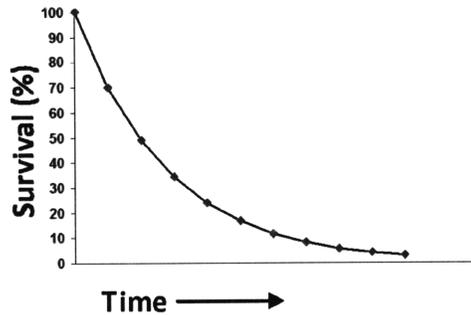
(A) Nearly all organisms show increased mortality rates (i.e. decreased survival) with increased age, as opposed to a fixed mortality rate (B) where death is equally likely at any age. (C) The mortality curves of divergent organisms closely resemble each other, suggesting a commonality in the aging process that might be amenable to scientific study in model organisms. Figure 1C is adapted from Greg Liszt, Molecular Studies of Longevity-Associated Genes in Yeast and Mammalian Cells, Graduate Thesis, MIT 2006. Reproduced here by permission.

Figure 1

A



B



C

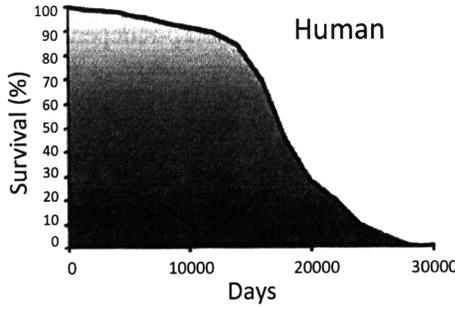
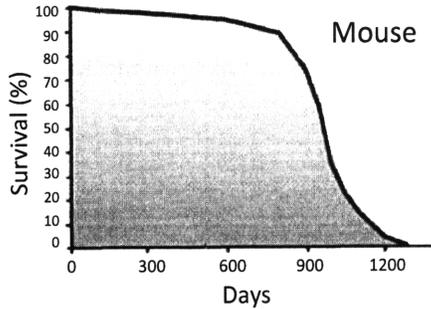
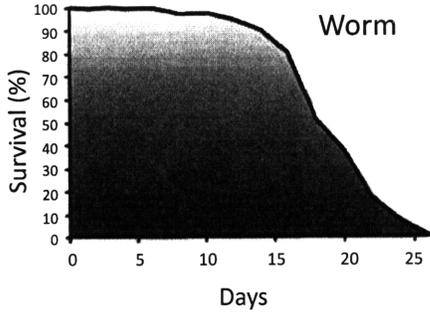
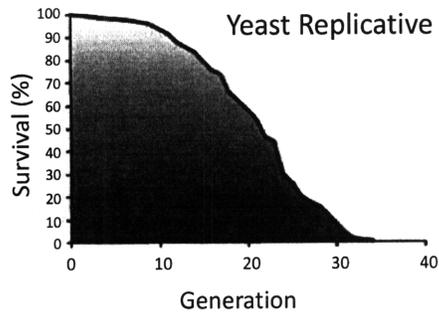


Figure 2. Molecular pathways mediating calorie restriction (CR) induced life extension in yeast.

Moderate CR in yeast (0.5% glucose) leads to activation of Sir2, HST2 and possibly other NAD-dependent Sirtuins by increasing NAD/NADH levels and reducing the Sir2 inhibitor, nicotinamide. This results in increased Sir2 enzymatic activity, which reduces the formation of toxic extrachromosomal ribosomal circles (ERCs) through silencing of the rDNA locus. A newer more 'extreme' form of CR, which utilizes 10 fold lower glucose levels (0.05% glucose), appears instead to require Target of Rapamycin (TOR) for life-extension in a yet unknown mechanism.

Figure 2

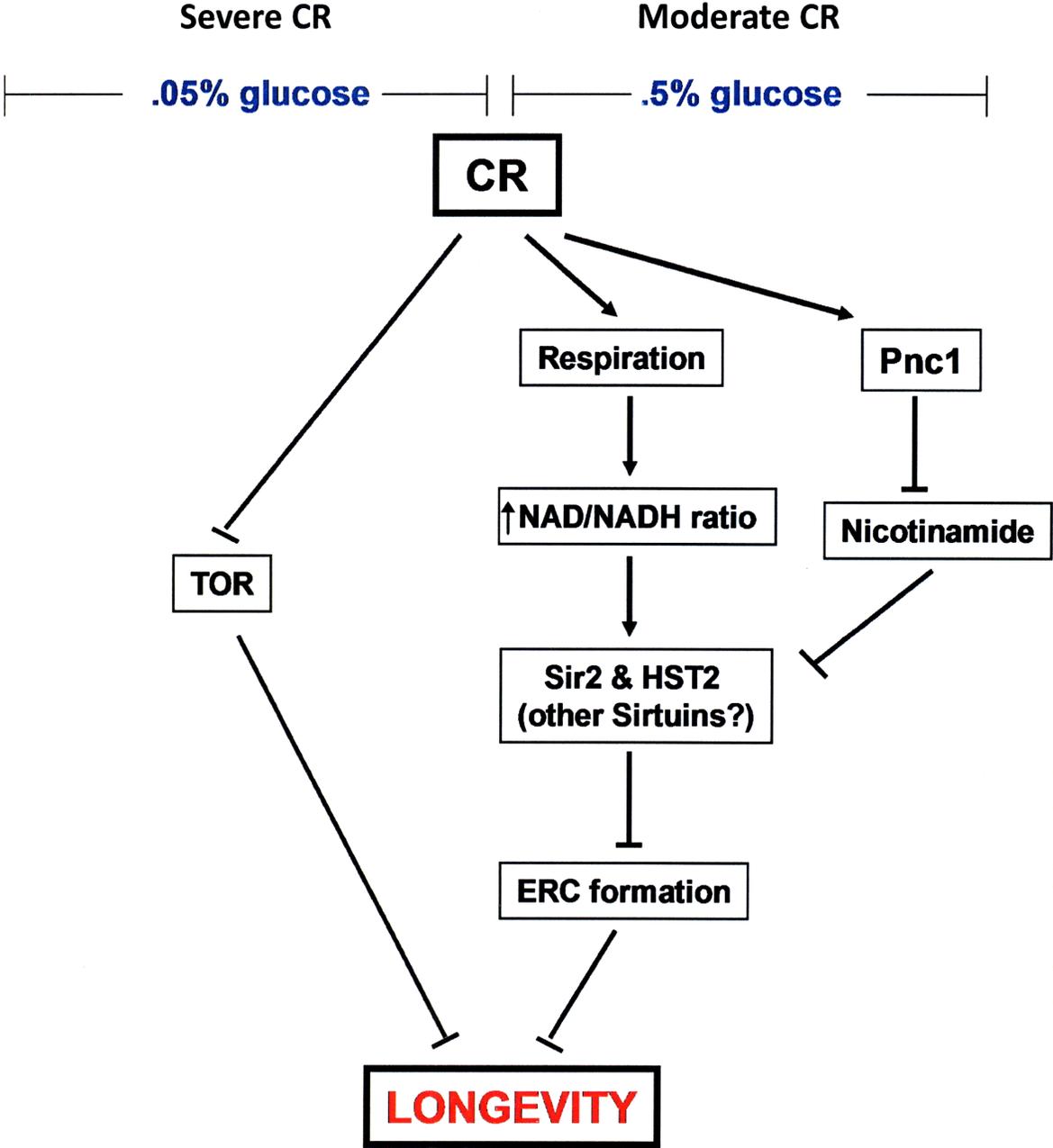


Figure 3. Sir2 mediates life extension in divergent organisms.

Overexpression of Sir2 has been shown to extend the lifespan of yeast, worms and flies (indicated by vertical black arrow). Since these organisms diverged more than a billion years ago, it suggests the ability of Sir2 to regulate lifespan existed in their last common ancestor. As a result, all descendants originating from this ancestor (including humans) are also thought to contain Sir2 proteins that regulate longevity. Adapted from Guarente and Picard, 2005.

Figure 3

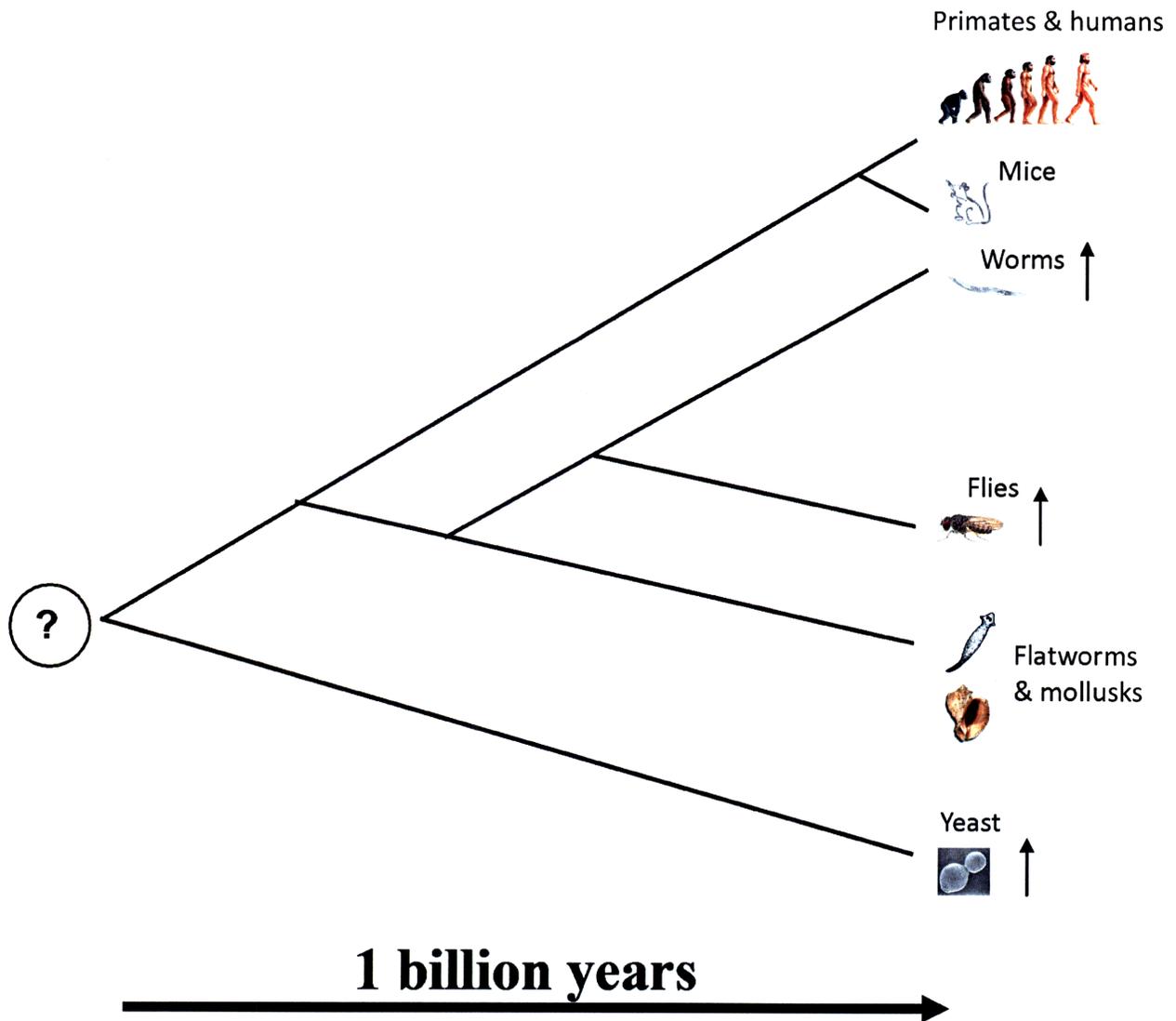
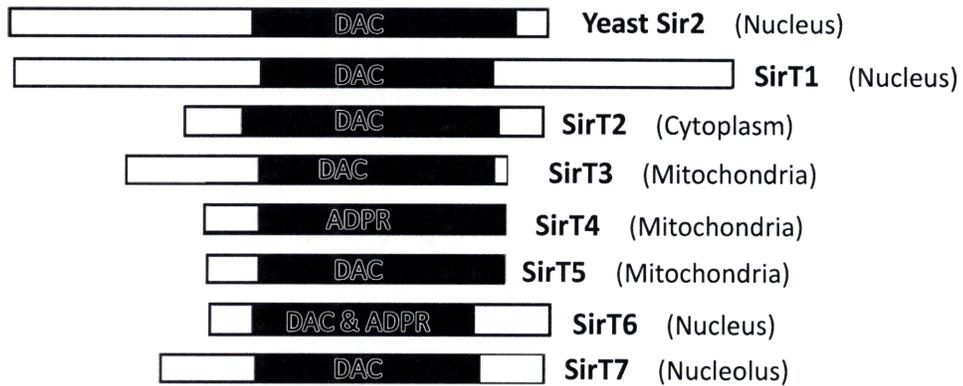


Figure 4. The Sirtuin family of deacetylases/ADP-ribosyltransferases.

(A) The mammalian Sirtuins all share a conserved core catalytic domain with yeast Sir2 (black bar), but have unique N-terminal and/or C-terminal sequences. (B) Sirtuins catalyze either a deacetylase (DAC) or a ADP-ribosyltransferase reaction (ADPR), both of which require NAD and give rise to nicotinamide (NAM). (C) An unrooted tree diagram derived from the phylogenetic analysis of the conserved domains of 60 Sirtuin sequences from all Sirtuin classes. The mammalian Sirtuins have been boxed. Figure 4A is adapted from Frye, 2000; Figure 4C is adapted from North and Verdin, 2004.

Figure 4

A



B



C

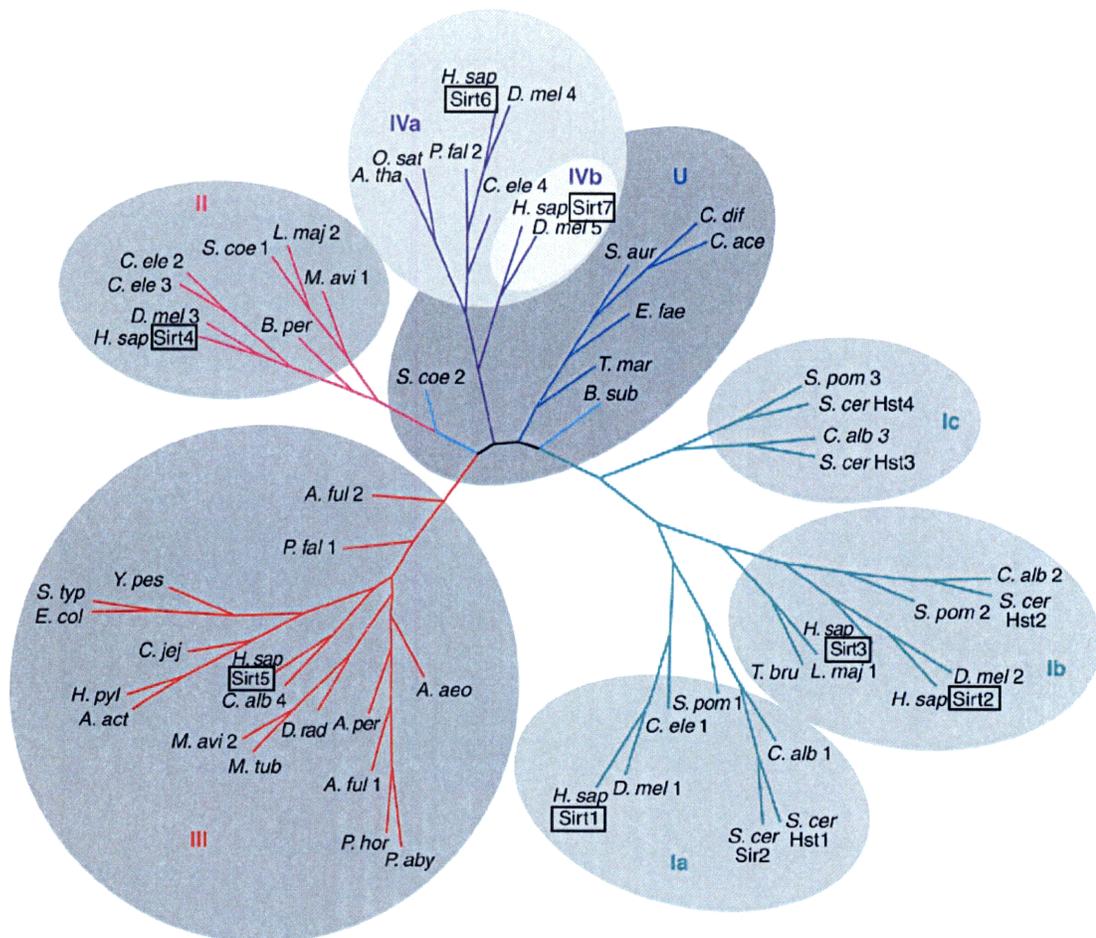


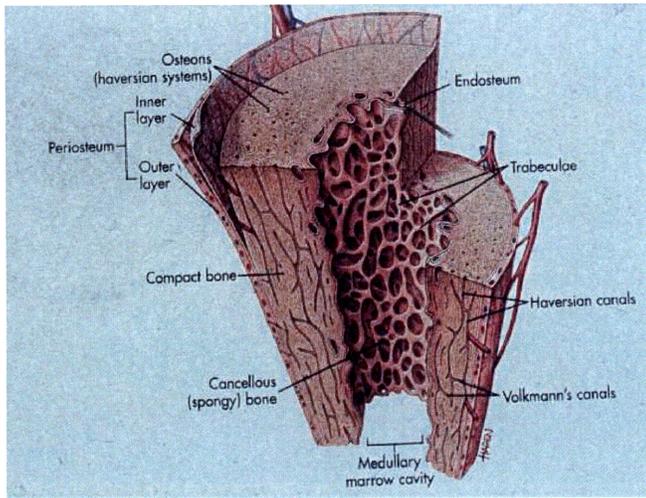
Figure 5. Anatomy of bone.

(A) A typical long bone contains a dense outer layer (cortical bone) and a more hollow inner layer (trabecular bone). (B) Osteoporosis is associated with the thinning of both the cortical and trabecular bone. (C) Osteoblasts are derived from pluripotent mesenchymal stem cells (MSCs) that also give rise to fat, cartilage, tendon, muscle and bone marrow stroma cells. (D) Osteoclasts are hematopoietic cells derived from a monocytic lineage that also gives rise to macrophages. Whereas MCSF (macrophage colony stimulating factor) is required for macrophage differentiation, both MCSF and RankL (receptor activator of NF κ B ligand) are required for osteoclast differentiation.

Figure 5A is adapted from <http://137.222.110.150/calnet/musculo/page3.htm>; 5B is adapted from Dempster et al., 1986; 5C is adapted from Minguell et al., 2001.

Figure 5

A



B

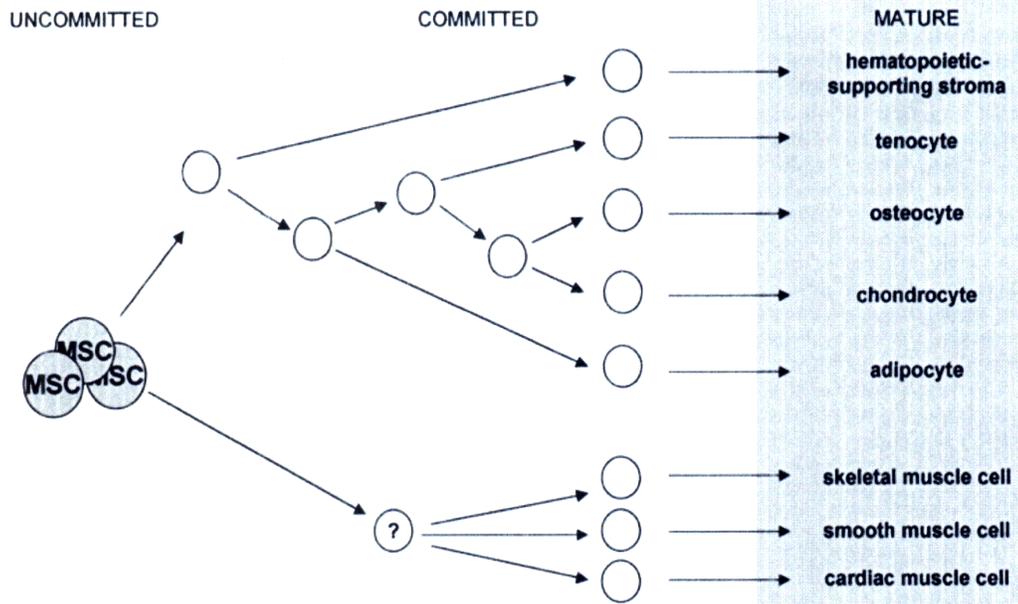
Normal bone



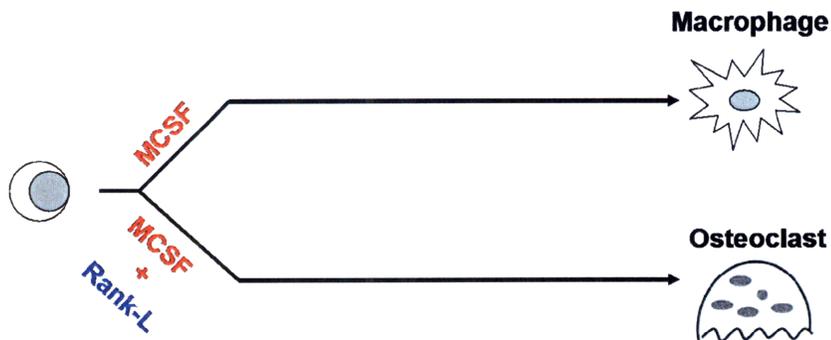
Osteoporotic bone



C



D



Chapter 2

Deletion of SirT1 causes an osteoporotic phenotype associated with decreased osteoblasts and increased osteoclasts

This chapter will be submitted for publication. The authors are Kayvan Zainabadi, James Edwards, Cassie Liu, Gregory Mundy, and Leonard Guarente. The author and James Edwards performed this work as an equally-shared, joint project; all mouse work was performed in the lab of Leonard Guarente, while all bone analyses were performed in the lab of Gregory Mundy.

SUMMARY

SirT1 is the mammalian orthologue of yeast Sir2, an NAD dependent histone deacetylase implicated in longevity. As mammals age, osteoblast numbers decrease while osteoclast numbers increase, resulting in age-related or post-menopausal osteoporosis. SirT1 has previously been shown to be a negative regulator of PPAR γ and NF κ B, two transcription factors that are important for the differentiation of osteoblasts and osteoclasts, respectively. Based on its known molecular targets, we hypothesized SirT1 might play a role in regulating bone mass *in vivo*. In support of this hypothesis, we observe significant bone deficiencies in SirT1 whole-body knockout mice, including reduced bone mineral density, cortical thickness and trabecular bone volume. These deficiencies are evident in mice as young as one month of age and worsen with age, suggesting a defect in bone remodeling. Consistent with this, knockout mice display a decrease in osteoblasts numbers and an increase in osteoclasts numbers per bone surface. We therefore conclude that SirT1 is an important regulator of bone mass, likely through regulation of osteoblast and osteoclast differentiation.

INTRODUCTION

The Sir2 family of proteins (Sirtuins) are NAD dependent deacetylases that are evolutionarily conserved from yeast to humans and regulate processes ranging from genomic silencing to metabolism (Haigis and Guarente, 2006). Sir2 uses NAD as a co-substrate to catalyze deacetylation of acetyl-lysine groups, giving rise to nicotinamide and o-acetyl-ADP-ribose as products (Imai et al., 2000). In yeast, increased dosage of Sir2 extends lifespan by deacetylating histones at ribosomal DNA loci, thereby reducing formation of toxic extrachromosomal ribosomal circles (ERCs) – a cause of aging in yeast (Sinclair and Guarente, 1997). Intriguingly, Sir2 orthologues in higher organisms also promote longevity, though in a mechanism that appears independent of ERCs (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001).

Another regiment that has been shown to extend life-span in a wide variety of organisms is calorie restriction (CR). In yeast, CR requires Sir2 for life-extension, and activates its enzymatic function by increasing NAD/NADH levels, as well as decreasing the Sirtuin inhibitor, nicotinamide (Lin et al., 2002; Anderson et al., 2003). Recent studies show that CR also induces expression of SirT1 (the mammalian orthologue of Sir2) in mammalian tissues as well (Cohen et al., 2004; Nisoli et al., 2005). Intriguingly, SirT1 knockout mice fail to display several phenotypes associated with CR, including increased activity, higher respiration and extension of lifespan (Chen et al., 2005; Boily et al., 2008).

The mammalian genome contains seven Sirtuins, of which SirT1 shares the greatest sequence similarity with yeast Sir2. SirT1 regulates numerous physiological processes, such as apoptosis, metabolism and cell differentiation, by deacetylating key transcription factors in each pathway (Fulco et al., 2003; Vaziri et al., 2001; Haigis and Guarente, 2006). For instance,

through repression of NF κ B, SirT1 sensitizes cancer cells to TNF α -induced apoptosis (Yeung et al., 2004); protects against microglia dependent amyloid- β toxicity (Chen et al., 2005); and lowers activation of pro-inflammatory pathways associated with aging (Pfluger et al., 2008).

Osteoporosis is a well established disease of aging that is characterized by low bone mass. It has two known causes: 1) upregulation of osteoclast activity (Type I, post-menopausal osteoporosis); and 2) downregulation of osteoblast activity (Type II, age-related osteoporosis). In each instance, the coupled process of bone remodeling (i.e. bone formation and bone resorption) becomes imbalanced, resulting in a gradual loss of bone.

Osteoblasts, the bone forming cells of the skeleton, are derived from a pluripotent mesenchymal stem cell (MSC) population that also gives rise to adipocytes, myocytes, and chondrocytes (Harada and Rodan, 2003). Incidentally, there appears to be an intrinsic competition between adipocytes and osteoblasts for access to this population: differentiation among these two lineages is mutually exclusive and differentiation down one lineage appears to inhibit the other. As mammals age, osteoblast numbers decrease while adipocyte numbers increase, resulting in an accumulation of fat in tissues that normally do not store fat, such as the bone marrow. Intriguingly, pharmacological activation of PPAR γ , the master fat transcription factor, also leads to a similar phenotype where the bone marrow becomes 'fatty' and osteoblast numbers decline (Rzonca et al., 2004). Inversely, PPAR γ +/- mice are not only resistant to obesity and diabetes but also have increased osteoblastogenesis and bone mass (Akune et al., 2004). These data suggest that misregulation in the proper balance between adipogenesis and osteoblastogenesis during the aging process (possibly through hyperactivation of PPAR γ) might be a cause of age-related osteoporosis. It is therefore interesting to note that SirT1 is an

endogenous repressor of PPAR γ , though its role in osteoblastogenesis has yet to be explored (Picard et al., 2004).

Osteoclasts, on the other hand, are hematopoietic cells derived from a monocytic lineage. Unlike MSCs, monocytes are only bipotential - capable of differentiating into either osteoclasts or macrophages (Teitelbaum, 2000). Activation of the NF κ B signaling pathway has been shown to be a pivotal step in osteoclasts differentiation: NF κ B turns on much of the early osteoclast transcriptional program and mutations in NF κ B result in osteopetrosis (increased bone mass) due to a block in osteoclastogenesis (Asagiri and Takayanagi, 2007). Since SirT1 is a known repressor of NF κ B signaling, we surmised it might also have a role in regulating osteoclast differentiation.

To determine the effects of SirT1 on bone mass, we have taken a mouse genetics approach. Analysis of SirT1 whole-body knockout mice reveals significant bone deficiencies which worsen with age. Interestingly, this osteoporotic phenotype is associated with decreased osteoblast, and increased osteoclast numbers, suggesting SirT1 regulates bone mass through regulation of osteoblast and osteoclast differentiation. SirT1 might, therefore, act as a novel factor linking nutrient conditions with bone remodeling.

MATERIALS AND METHODS

Animal experimentation

All mice were housed under controlled temperature (25 \pm 1 $^{\circ}$ C S.D.) and lighting conditions. Food provided was normal chow. Mice were cared for in accordance with the MIT animal care committee.

Microcomputed tomography (micro-CT) and X-ray analysis

Dissected bones were analysed for overall mineral content by X-ray (Faxitron). Tibia and femur were analyzed by microCT scanning (uCT40, Scanco) at an isotropic voxel size of 12um (resolution 280). After the growth plate was identified in each scan set, the metaphyseal region 200um below this area was scanned and analyzed for alterations in trabecular bone parameters. Also, a 200um region of femoral diaphysis in the exact center of each bone was calculated, scanned and assessed for alterations in cortical bone dimensions.

Histologic and histomorphometric analyses

Lumbar vertebrae and long bones were collected following sacrifice and fixed for up to 48 hours in 10% formalin (Fisher). Undecalcified regions of vertebrae were processed and embedded in a methylmethacrylate-based resin (Sigma) and sectioned at 5µm. Sections were deplastasized and stained for bound calcium ions using the Von Kossa procedure with a van Gieson counterstain, or using a post-coupling staining technique for tartrate-resistant acid phosphatase. Bone volume and cellular distribution was quantified histomorphometrically using Osteomeasure quantification software (Osteometrics). Osteoblasts and osteoclasts were identified based on both morphology and staining with alkaline phosphatase or tartrate resistant acid phosphatase (TRAP), respectively. Bone formation rate was determined by double-labeling with calcein injection at 4-day intervals, with animals sacrificed on the sixth day.

Statistical analysis

Analysis was performed using an unpaired Student's t-test, with p-values less than 0.05 considered significant. All data is presented \pm standard error of the mean (SEM).

RESULTS

SirT1 knockout mice show reduced bone mineral density

A mouse knockout model of SirT1 has previously been generated that results in the complete absence of the SirT1 protein (**Figure 1A**) (McBurney et al., 2003). Interestingly, knockout embryos have been reported to display instances of exencephaly, a lethal developmental defect where the skull fails to fully enclose the brain. Newborn pups also show delays in the closure of craniofacial sutures and in the mineralization of the skull, vertebrae and digits (McBurney et al., 2003). These data suggest SirT1 plays a role in bone development, which has previously been attributed to reduced IGF signaling (Lemieux et al., 2005). However no studies yet have assessed the role of SirT1 in the regulation of adult bone mass.

SirT1 knockout mice display a number of abnormalities, including delays in eye-opening, sterility, and reduced body weight (**Figure 1B**). To determine if they had any bone deficiencies, we performed X-ray and bone mineral density (BMD) scans on adult animals (10 months old). Gross X-ray analysis reveals knockout mice have a readily discernible reduction in bone density (**Figure 2A**). BMD scans confirm the osteoporotic phenotype: knockout mice display a 46% reduction in tibia BMD; a 32% reduction in femur BMD; and a 29% reduction in whole-body BMD (**Figure 2B**). These data suggest that SirT1 is required for the proper maintenance of adult bone mass.

SirT1 knockouts have reduced cortical and trabecular bone volume

We were next interested in determining whether the decreased bone mass was due to reduced cortical or trabecular bone volume. We therefore performed microcomputed tomography (micro-CT) to create a three dimensional reconstruction of either the femoral head (trabecular bone) or femoral shaft (cortical bone). Gross micro-CT scans show a marked reduction in the total amount of mineralized tissue occupying the bones of knockout animals (**Figure 3A**).

Further, quantitation of each bone region demonstrates that knockout mice have a 44% reduction

in the amount of trabecular bone and a 47% reduction in the amount of cortical bone in knockout animals (**Figure 3B**). These results confirm an osteoporotic phenotype in the knockout mice that closely resembles human osteoporosis.

To further characterize this phenotype, we next analyzed the bone mass of both young and adult knockout mice. Micro-CT scans show that knockout animals as young as 1 month have a significant reduction in bone volume (27%), which increases to 51% at 4 months (**Figure 4**). Further, SirT1 heterozygous mice (+/-) have bone values indistinguishable from wildtype mice, suggesting haplosufficiency of SirT1. The progressive nature of the bone loss suggests a premature osteoporotic phenotype that is likely characterized by an imbalance in bone remodeling.

Histological analysis

To characterize the phenotype at a molecular level, we performed histological analysis on the L3-4 vertebrae of wildtype and knockout mice. Gross histology confirms a clear reduction in the amount of mineralized bone in the vertebrae of knockout animals (**Figure 5A**). We next performed cellular counts on histological sections to determine the numbers of osteoblasts and osteoclasts per bone surface. Intriguingly, knockout mice show both a 39% reduction in the number of osteoblasts and a 27% increase in the number of osteoclasts (**Figure 5B**). Consistent with this, knockout mice show a deficiency in the ability to make new bone, as assayed by bone formation rate (BFR). These results confirm that knockout animals have a defect in bone remodeling, thereby implicating SirT1 as a novel regulator of bone mass. Interestingly, knockout mice show characteristics of both Type I and Type II osteoporosis, suggesting SirT1 regulates the differentiation of both osteoblasts and osteoclasts.

DISCUSSION

Several lines of evidence have suggested that SirT1 might play a role in the maintenance of adult bone mass. First, SirT1 knockout pups have delays in the mineralization of their appendages and skulls (McBurney et al., 2003). Second, SirT1 is a known repressor of NF κ B and PPAR γ , which are known to be important in osteoclast and osteoblast differentiation, respectively (Yeung et al., 2004; Picard et al., 2004). Finally, mice treated with the SirT1 agonist, resveratrol, display increased bone mass (Pearson et al., 2008). In this study we show that SirT1 is in fact an important regulator of bone mass *in vivo*. SirT1 knockout mice display significant bone deficiencies at one month of age, suggesting a defect in bone modeling. Further, the phenotype appears to worsen with age, suggesting a defect also in bone remodeling. Consistent with this, SirT1 knockout mice show both a decrease in osteoblast numbers and an increase in osteoclast numbers. These results therefore suggest that SirT1 controls bone mass through the activation of osteoblasts and inhibition of osteoclasts.

With the mounting evidence linking SirT1 with longevity, these results provide an intriguing link between SirT1 and the classic aging disease, osteoporosis. Interestingly, SirT1 activation with resveratrol has been shown to extend the lifespan of lower organisms, including the vertebrate fish species, *Nothobranchius furzeri*, and mice fed a high-fat diet (Howitz et al., 2003; Valenzano et al., 2006; Baur et al., 2006). And while detailed lifespan studies do not currently exist for SirT1 transgenic mice, they do appear to display a number of characteristics associated with longevity. These include increased resistance to obesity, metabolic disorders (including diabetes), and inflammatory diseases (Banks et al., 2008; Pfluger et al., 2008). However, none of these mice have been analyzed to determine if they are protected against osteoporosis.

In contrast to SirT1 transgenics, SirT1 knockout mice display a variety of developmental disorders that result in reduced viability (McBurney et al., 2003). These include sterility (McBurney et al., 2003); metabolic disorders including hyperphagia and glucose insensitivity (Boily et al., 2008); behavioral defects such as abnormal circadian rhythm (Boily et al., 2008); and possible predisposition to autoimmune disease (Sequeira et al., 2008). In addition, the mice have also been reported to have decreased IGF1 signaling owing to the overexpression of the inhibitor IGF binding protein, IGFBP1 (Lemieux et al., 2005). Given these pleiotropic effects, it is hard to pinpoint whether the osteoporotic phenotype is due to cell autonomous or cell non-autonomous effects of SirT1 (though evidence will be presented in later chapters suggesting a cell autonomous role for SirT1 in osteoblasts and osteoclasts).

Regardless of its mode of action, our results paint a clear picture that SirT1 plays an important role in regulating bone homeostasis *in vivo*. Given SirT1 knockouts display aspects of both post-menopausal and age-related osteoporosis, it will be interesting to determine whether small molecule activators of SirT1 might serve as useful therapies for the treatment of both causes of osteoporosis (Milne et al., 2007). Recent evidence suggests that there is ample reason to be optimistic – mice treated with resveratrol have been shown to be more resistant against age-related osteoporosis (Pearson et al., 2008).

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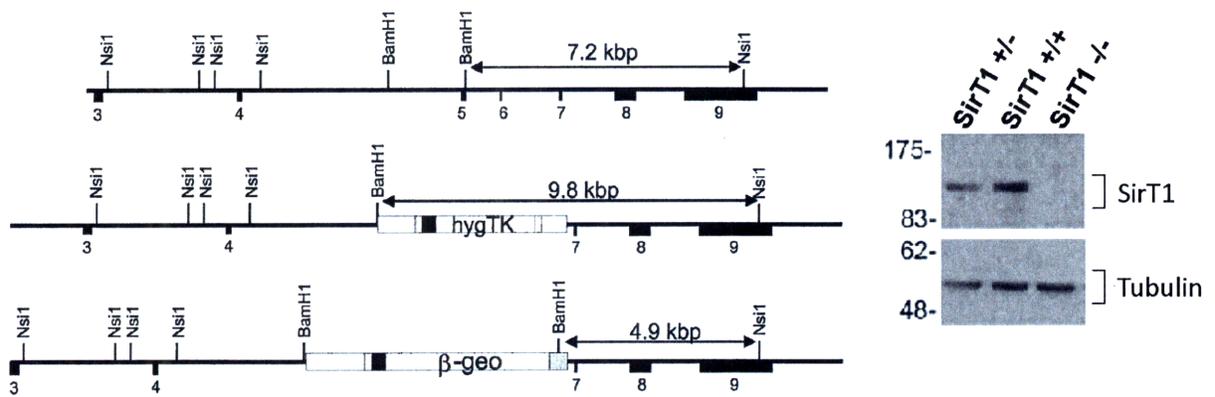
Figure 1. SirT1 whole-body knockout mice.

(A) A mouse knockout model of SirT1 has been previously generated through homologous recombination which results in the complete lack of SirT1 protein in all tissues (McBurney et al., 2003).

(B) SirT1 knockout animals can be obtained at sub-Mendelian ratios in an outbred background. Knockout mice display a number of abnormalities, including delays in eye-opening, sterility, and reduced body weight. Error bars represent standard error of the mean (SEM). (n = 8 for each group; * p<.05; ** p<.01; *** p<.005).

Figure 1

A



B

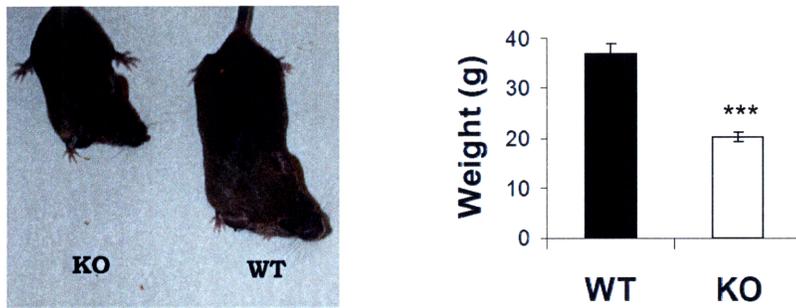


Figure 2. SirT1 knockout mice display reduced bone mineral density (BMD).

(A) SirT1 knockout mice have decreased bone density that is readily discernible by X-ray analysis.

(B) Knockout mice display a 46% reduction in tibia BMD, a 32% reduction in femur BMD, and a 29% reduction in whole-body BMD. Error bars represent SEM. ($n \geq 6$ for each group; * $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 2

A



B

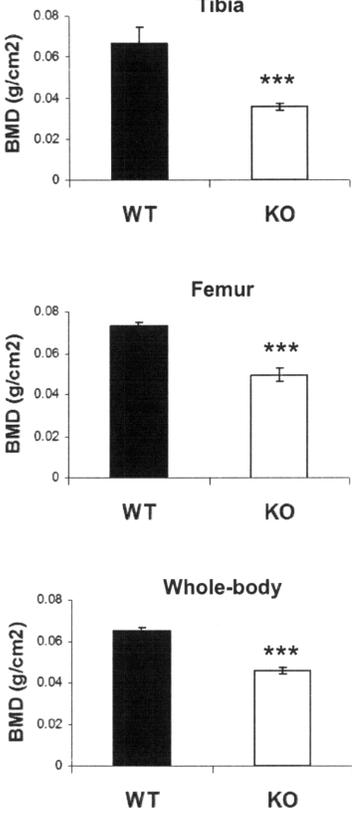


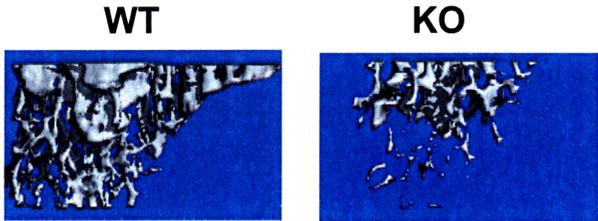
Figure 3. SirT1 knockouts display reduced cortical thickness and trabecular bone volume.

(A) Microcomputed tomography (micro-CTt) scans show reduced bone volume in SirT1 knockout animals.

(B) SirT1 knockouts display a 47% decrease in cortical thickness and 44% decrease in trabecular bone volume/total volume (BV/TV). These deficiencies mirror what is commonly observed in human osteoporosis. Error bars represent SEM. (n \geq 6 for each group; * p<.05; ** p<.01; *** p<.005).

Figure 3

A



B

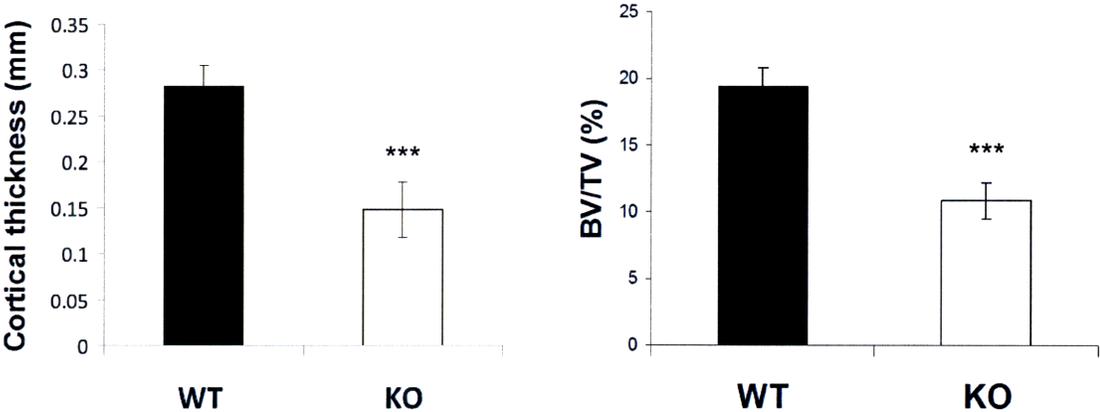


Figure 4. The osteoporotic phenotype of SirT1 knockouts worsens with age.

SirT1 knockout animals show a 27% reduction bone volume/total volume (BV/TV) at 1 month of age, which increases to 51% at 4 months. The progressive nature of the bone loss suggests an imbalance in bone remodeling that is often associated with human aging. Error bars represent SEM. (n \geq 5 for each group; * p<.05; ** p<.01; *** p<.005).

Figure 4

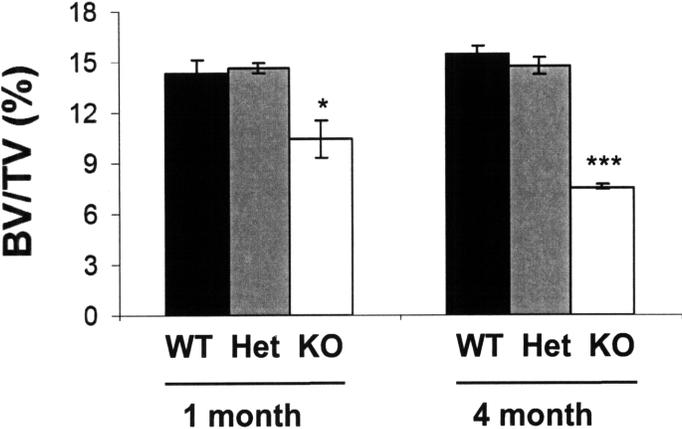


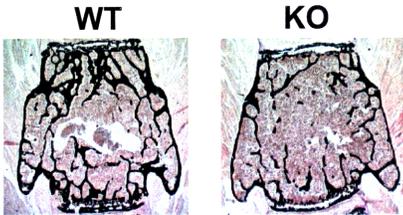
Figure 5. SirT1 knockout mice display decreased osteoblast numbers and increased osteoclast numbers.

(A) Gross histology shows a clear reduction in the amount of mineralized bone occupying the L3-L4 vertebrae of knockout mice (as assayed by Von Kossa staining; black indicates mineralized bone).

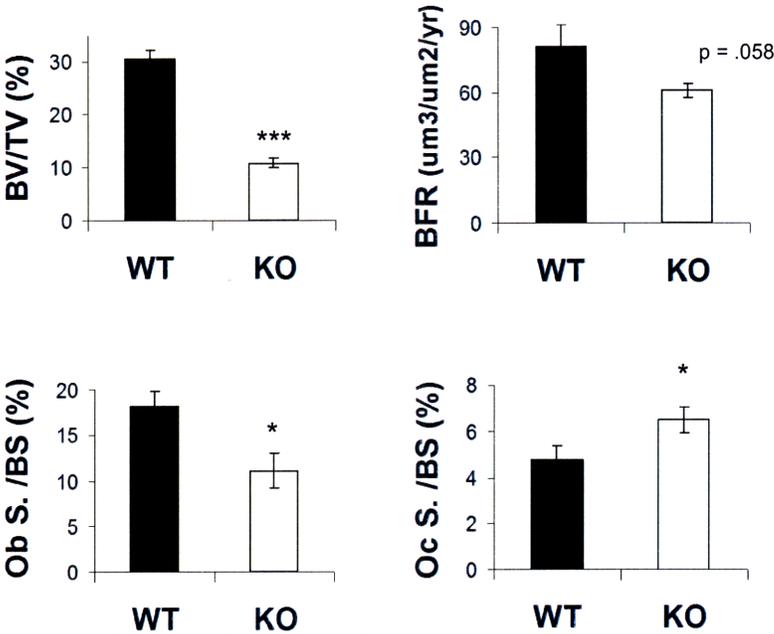
(B) Histomorphometry shows that the reduction in bone volume (BV/TV) is accompanied by a decrease in osteoblast numbers per bone surface (Ob. S./BS) and an increase in osteoclast numbers per bone surface (Oc. S./BS). Consistent with this, knockout mice show a reduced ability to form new bone as assayed by bone formation rate (BFR). Error bars represent SEM. ($n \geq 6$ for each group; * $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 5

A



B



Chapter 3

Sirt1 deletion in osteoblasts causes an osteoporotic phenotype associated with decreased osteoblasts and Runx2 hypoactivity

This chapter will be submitted for publication. The authors are Kayvan Zainabadi, James Edwards, Cassie Liu, Gregory Mundy, and Leonard Guarente. The author and James Edwards performed this work as an equally-shared, joint project; all mouse work and cell experiments was performed in the lab of Leonard Guarente, while all bone analyses were performed in the lab of Gregory Mundy.

SUMMARY

In Chapter 2, we showed that SirT1 whole-body knockout mice have an osteoporotic phenotype associated with decreased osteoblast and increased osteoclast numbers. Here, we have used Cre/Flox technology to create osteoblast specific SirT1 knockout mice (ObKO) using the 2.3kb Collagen type I promoter. Unlike SirT1 whole-body knockouts, ObKO mice display no gross developmental defects and are indistinguishable from wildtype mice. However, similar to the whole-body knockouts, ObKOs show an osteoporotic phenotype associated with reduced osteoblast numbers. Interestingly, this phenotype is only found in adult animals, suggesting a defect specifically in bone remodeling. We find that SirT1 stimulates osteoblast differentiation through a novel interaction – by binding to and activating Runx2, a master osteoblast transcription factor. Osteoblasts lacking SirT1 show decreased expression of Runx2 targets, while cells treated with SirT1 agonists show the inverse. Interestingly, we find a similar induction of Runx2 targets in wildtype mice treated with resveratrol, a small molecule activator of SirT1 that has recently been shown to lead to increases in bone mass. All in all, our results indicate that SirT1 is an important regulator of bone mass through cell autonomous regulation of osteoblast differentiation. SirT1 might therefore serve as an ideal pharmacological target for the development of anabolic drugs to treat age-related osteoporosis.

INTRODUCTION

Bone consists of two different types of cells: the mesenchymal derived osteoblasts that form bone, and the haematopoietic derived osteoclasts that break down bone. Osteoblasts and osteoclasts work together to continuously replace old bone with new bone. This coupled process, called bone remodeling, occurs throughout life and is essential for the maintenance of bone strength and resilience. Osteoporosis occurs when this process becomes uncoupled, either through upregulation of osteoclast activity (Type I, post-menopausal osteoporosis) or downregulation of osteoblast activity (Type II, age-related osteoporosis).

Osteoblasts are derived from a mesenchymal pluripotent stem cell population in the bone marrow that also gives rise to adipocytes, myocytes, and chondrocytes (Harada and Rodan, 2003). Differentiation of a mesenchymal stem cell down any lineage involves two steps: first, commitment to a particular fate which results in a cycling progenitor cell (i.e. a pre-osteoblast); and second, differentiation of the progenitor cell to a terminal cell type (i.e. from pre-osteoblast to osteoblast). In this regard, SirT1 has been shown to inhibit terminal differentiation of pre-adipocytes to adipocytes and myoblasts to myocytes, and promote differentiation of chondroblasts to chondrocytes (Picard et al., 2004; Fulco et al., 2003; Dvir-Ginzberg et al., 2008). However, its role in differentiation of osteoblasts has yet to be determined.

Bone development is a complex process whose molecular players have only recently been identified. The Runx2 transcription factor has been shown to be essential for bone formation – Runx2 knockout mice lack a mineralized skeleton, and overexpression of Runx2 is sufficient to activate the osteoblast transcriptional program (Ducy et al., 1993; Komori et al., 1993; Otto et al., 1993). Runx2 mediates its effects by binding to specific cis-acting elements (OSE2) in the promoter of nearly all of the major osteoblast genes, including Osteocalcin,

Osteopontin, and Bone Sialoprotein (Harada and Rodan, 2003; Ducky et al., 1993). However, recent data has suggested the existence of additional factors important for osteoblastogenesis.

This hypothesis was confirmed with the cloning of a second essential osteoblast transcription factor, Osterix (*Osx*) (Nakashima et al., 1997). Much like *Runx2*, *Osx* knockout mice lack a mineralized skeleton, and overexpression of *Osx* is sufficient to induce expression of osteoblast markers. Since *Osx* is not expressed in *Runx2* knockouts, while *Runx2* is expressed in *Osx* knockouts, *Osx* is commonly placed downstream of *Runx2*. Consistent with this, OSE2 elements have been described in the promoter region of *Osx*, and overexpression of *Runx2* has been shown to induce expression of *Osx* (Nishio et al., 2006).

While *Runx2* and *Osx* work together to promote terminal differentiation of osteoblasts, there is evidence that other signaling pathways mediate the earlier events of osteoblastogenesis. For instance, commitment of the earlier pluripotent mesenchymal stem cell (MSC) is controlled by the activation of other master transcriptional regulators (Harada and Rodan, 2003). Within this context, it has been shown that PPAR γ activation can repress commitment of the MSC towards the osteoblast lineage (Rzonca et al., 2004). However, PPAR γ appears to have little, if any effect, in cells already committed to the osteoblast lineage (Kawaguchi et al., 2005). Interestingly, SirT1 is a known repressor of PPAR γ (Picard et al., 2004).

The bone morphogenetic proteins (BMPs), particularly BMP2, also appear as important factors for the early steps of osteoblastogenesis. BMP2 has been shown to upregulate transcription and activity of both *Runx2* and *Osx*, largely through the SMAD signaling pathway (Hong et al., 2005; Chen et al., 2004). Moreover, certain SMADs have also been shown to directly bind RUNX2 and activate transcription of its downstream genes. Recent evidence suggests that BMP2 also mediates its effects through the homeobox family of transcription

factors, including Msx2 (Msh homeobox homolog), Dlx3 and Dlx 5 (distal-less homeobox) (Marie, 2008). All three are induced in response to BMP2 and appear important for BMP2 mediated transcriptional activation of Runx2 and Osx (Matsubara et al., 2008; Hassan et al., 2004; Harada and Rodan, 2003).

Besides transcriptionally, Runx2 activity has also been shown to be regulated at a post-translational level. For example, Runx2 is phosphorylated and thereby activated by the MAPK (mitogen-activated protein kinase) signaling pathway (Komori, 2008). Interestingly, various members of the class I and class II histone deacetylases (HDAC) are also known to interact with Runx2 and repress its activity (Jensen et al., 2007). For instance, HDAC3 and HDAC6 bind to, deacetylate and repress Runx2-mediated transcription (Schroeder et al., 2004); HDAC4 binds to Runx2 and interferes with its DNA binding ability (Jeon et al., 2006); HDAC5 deacetylates Runx2 and thereby promotes its ubiquitin-mediated proteolysis (Kang et al., 2005); and finally, HDAC7 represses Runx2 transcriptional activity through a poorly understood deacetylase-independent mechanism (Jensen et al., 2008).

Here we present evidence that SirT1, a member of the class III deacetylase family, is unique in that it functions to enhance Runx2 activity. This activation has important physiological consequences: mice lacking SirT1 specifically in their osteoblasts develop an osteoporotic phenotype due to decreased osteoblastogenesis. Inversely, we find an induction of Runx2 targets in mice treated with resveratrol, a small molecule activator of SirT1 that has recently been shown to protect against aging associated bone loss (Pearson et al., 2008). Our results therefore paint SirT1 as a novel positive regulator of bone mass through its cell autonomous regulation of osteoblast differentiation.

MATERIALS AND METHODS

Animal experimentation

All mice were housed under controlled temperature ($25 \pm 1^\circ\text{C}$ S.D.) and lighting conditions. Food provided was normal chow. Mice were cared for in accordance with the MIT animal care committee.

Microcomputed tomography (micro-CT) and X-ray analysis

Tibia and femur were analyzed by microCT scanning (uCT40, Scanco) at an isotropic voxel size of 12 μm (resolution 280). After the growth plate was identified in each scan set, the metaphyseal region 200 μm below this area was scanned and analysed for alterations in trabecular bone parameters. Also, a 200 μm region of femoral diaphysis in the exact center of each bone was calculated, scanned and assessed for alterations in cortical bone dimensions.

Histologic and histomorphometric analyses

Lumbar vertebrae and long bones were collected following sacrifice and fixed for up to 48 hours in 10% formalin (Fisher). Undecalcified regions of vertebrae were processed and embedded in a methacrylate-based resin (Sigma) and sectioned at 5 μm . Sections were deplastasized and stained for bound calcium ions using the Von Kossa procedure with a van Gieson counterstain, or using a post-coupling staining technique for tartrate-resistant acid phosphatase. Bone volume and cellular distribution was quantified histomorphometrically using Osteomeasure quantification software (Osteometrics). Osteoblasts and osteoclasts were identified based on both morphology and staining with alkaline phosphatase or tartrate resistant acid phosphatase (TRAP), respectively. Bone formation rate was determined by double-labeling with calcein injection at 4-day intervals, with animals sacrificed on the sixth day.

Isolation and differentiation of primary osteoblasts

Primary osteoblast precursors were isolated from 1-3 day old pups. Calvaria were removed and placed in 5mL of no-serum α -MEM media solution containing of 0.1% Collagenase type 2 and 0.1% Trypsin/EDTA. Eight incubations were performed at 37°C for 15' in an orbital shaker, with the first two discarded and the remaining collected on ice, spun down and plated in 10% α -MEM (3 pups/10cm plate). Cells were expanded for a maximum of three passages, then trypsinized, filtered through a 70 μ m nylon filter (BD Falcon), and plated for experiments.

To minimize any extraneous effects on proliferation, cells were infected only upon confluence and immediately preceding differentiation. To excise SirT1, cells were treated with either CRE adenovirus or empty vector (as a control) at 50 MOI for 24 hours in α -MEM containing 10% FBS. Cells were then allowed to recover for 24 hours before being differentiated (Day 0) with 50ug/mL ascorbic acid and 10mM β -glycerophosphate. In experiments with SRT1720 and SRT2183 (SirTris), drugs were added at 1 μ M (unless otherwise stated) at Day 0 and DMSO was used as empty vehicle control.

Alkaline phosphatase and alizarin red staining

Vector and Cre-infected cells were stained for alkaline phosphatase and mineralization at various points of differentiation. Cells were washed twice with 1X Tris-buffered saline (TBS), then fixed with 10% neutral buffered formalin for 10'. Alkaline phosphatase was stained using Alkaline Phosphatase Blue Membrane Substrate kit (Sigma) according to manufacturer's instructions. Mineralization was stained using 1% Alizarin Red (VWR) for 5-10'. After staining, cells were extensively washed with TBS and finally with water.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells or tissues using TRIZOL (Invitrogen) and cleaned-up with the RNeasy MinElute Cleanup kit (Qiagen). For calvaria preps, non-osseous tissue was first removed in ice-cold PBS, then calvaria was minced in Trizol, and homogenized using a Tissue Tearor homogenizer (VWR). Lysates were then spun down at 15,000g for 10minutes, with the resulting supernatant used for RNA isolation according to manufacturer's instructions.

1 ug of cDNA was synthesized from total RNA by first-strand synthesis with random hexamers using SuperScript III reverse transcriptase (Invitrogen). cDNA was then subjected to realtime-PCR analysis with gene-specific primers in the presence of iQ SYBR green (Bio-Rad) (**Table 1**). Depending on the abundance of target transcript, stock cDNA was diluted anywhere in the range of 1:3 to 1:20 to achieve optimal crossing point (CP) values. Relative mRNA abundance was obtained by normalization to Rpl19 levels. The protocol for qRT-PCR is as follows: the cDNA was denatured by heating the reaction to 95°C for 5', then 45 cycles at 95°C for 10", 55°C for 15", and 72°C for 25". After amplification, a melting curve analysis was taken to ensure the presence of only one amplification product.

Western blot, immunoprecipitation (IP), and luciferase assays

Antibodies were obtained from the following sources: SirT1 (Upstate), Runx2 (Sigma, Abcam), HSP90 (Abcam), SirT6 (Cell Signalling), Flag (Sigma), HA (Santa Cruz). Immunoprecipitations (IPs) were carried out in the following manner: 15 cm plates were washed twice with PBS, scraped in presence of PBS + 0.5% Triton X-100 (Sigma) including complete protease inhibitors (Roche) and homogenized by passing (5 times each) through a 21G needle, 23 G, and finally a 26G. Cells were then allowed to lyse on an orbital shaker for 30 minutes, then spun down at 15,000g for 10 minutes, with the resulting supernatants used for experiments. IPs were generally performed using antibodies at a final concentration of 5ug-10ug/ml overnight at

4°C. Protein G agarose (Santa Cruz biotechnology) was then added for 1-2 hours, and then washed at least 3 times with PBS + 0.1% Triton, and boiled for 3 minutes in SDS sample buffer. Antibody controls were performed in order to distinguish between IgG heavy chain which consisted of IPs using lysis buffer alone. To separate Runx2 from the heavy chain, 4-15% gradient gels were used and run at 40 milliAmps for 2 hours. Luciferase assays were performed as described in Hong et al., 2005.

Statistical analysis

Analysis was performed using an unpaired Student's t-test, with p-values less than 0.05 considered significant. All data is presented \pm standard error of the mean (SEM).

RESULTS

Generation of Osteoblast specific SirT1 knockout (ObKO) mice

In Chapter 2, we demonstrated that whole-body SirT1 knockout mice display an osteoporotic phenotype that is, in part, due to reduced osteoblast numbers. To gain further insight into the role of SirT1 in osteoblast differentiation, we generated osteoblast specific knockouts by crossing SirT1 floxed mice with mice expressing Cre under the 2.3kb Collagen type 1 promoter (**Figure 1A**) (Cheng et al., 2003; Liu et al., 2004; Dacquin et al., 2002). This promoter, which has been used extensively before, is specific to osteoblast progenitors that have exited the cell cycle and are undergoing differentiation (Dacquin et al., 2002; Lee et al., 2007). Therefore, excision of SirT1 would be predicted to specifically affect differentiation, and not proliferation, of osteoprogenitor cells. Expression of Cre leads to excision of the catalytic domain of SirT1, rendering it functionally dead (**Figure 1A**) (Cheng et al., 2003).

ObKO mice show an osteoporotic phenotype associated with reduced osteoblast numbers

Unlike SirT1 whole-body knockout mice, osteoblast knockout (ObKO) mice display no developmental defects and are indistinguishable from their wildtype littermates (**Figure 1B**). Microcomputed tomography (micro-CT) reveals ObKOs have normal bone mass at 1 month, but show a 32% reduction in bone volume/total volume (BV/TV) at 4 months of age (**Figure 2A**). The adult-onset of the phenotype suggests an imbalance in bone remodeling, reminiscent of what is commonly seen in human osteoporosis.

Histological examination confirms a reduction in the amount of mineralized bone volume in 4 month old, but not 1 month old, ObKOs (**Figure 2B**). Importantly, the osteoporotic phenotype in 4 month old ObKOs is associated with a decrease in osteoblast numbers, but not that of osteoclasts. Consistent with this, 4 month old ObKOs show a decreased ability to make new bone, as measured by bone formation rate (BFR) (**Figure 2B**). Since ObKOs are missing SirT1 specifically in their osteoblasts, these results indicate that SirT1 is required cell autonomously for proper osteoblast differentiation and function.

Osteoblasts lacking SirT1 show reduced expression of Runx2 targets

To gain molecular insight into how SirT1 promotes osteoblast differentiation, we isolated primary osteoblasts from SirT1 flox/flox mice (**Figure 3**). Since the 2.3kb Collagen type 1 promoter has previously been shown to be expressed only *in vivo*, we excised SirT1 instead with addition of a Cre-adenovirus (**Figure 4A**). To minimize any extraneous effects on cell proliferation, we infected cells only upon confluency and immediately preceding differentiation. Mirroring the *in vivo* results, Cre-infected cells show a marked reduction in both early and late markers of differentiation, including alkaline phosphatase and mineralization, respectively (**Figure 4B,C**).

Quantitative RT-PCR analysis reveals that this reduction is not due to hyperactivation of PPAR γ : PPAR γ itself is expressed at undetectable levels in these cells, and its downstream targets, Ap2 and Lipoprotein lipase, are not induced in Cre-infected cells. In addition, Msx2, Dlx3 and Dlx5, which are important for the early steps of differentiation, are also not different (**Figure 4D**). Rather, knockout cells show a two fold reduction in the expression of the major osteoblast transcription factor, Osterix (Osx); while Runx2 itself appears changed (**Figure 4E**). Since Osx is a downstream target of Runx2, we next examined expression of other Runx2 downstream targets, including Osteocalcin, Osteopontin, and Bone Sialoprotein (BSP). Interestingly, all three targets also show marked reductions in Cre-infected cells, suggesting hypoactivity of Runx2 (**Figure 4F**).

SirT1 interacts with and activates Runx2 transcriptional activity

Since Runx2 expression itself was not changed, but expression of its downstream targets were, we hypothesized SirT1 promoted the transactivation potential of the Runx2 protein. Consistent with this, tagged versions of SirT1 and Runx2 were found to interact in 293T cells: Flag-SirT1 was able to pull down HA-Runx2 and vice versa (**Figure 5A**). To determine whether SirT1 and Runx2 interacted at endogenous levels, we first pre-screened a number of commercially available antibodies for their ability to immunoprecipitate a tagged version of Runx2. From these experiments, we identified two antibodies (from Sigma and Abcam) that were able to efficiently immunoprecipitate endogenous Runx2 from whole-cell lysates of U2OS osteosarcoma cells (**Figure 5B**). Importantly, both antibodies were also found to co-immunoprecipitate SirT1, but not the closely related nuclear Sirtuin, SirT6, nor the abundantly expressed HSP90 protein. Therefore, we conclude that Runx2 specifically interacts with SirT1 in osteoblast cells.

Next, to determine the molecular consequences of this interaction, we used a Runx2 luciferase reporter (p6OSE2) which contains six Runx2 specific OSE2 elements upstream of luciferase. As expected, we saw a dose-dependent increase in luciferase activity with increased dosage of the construct, confirming that endogenous Runx2 is able to activate the promoter in U2OS cells. Overexpression of SirT1 significantly increased this luciferase activity, whereas RNAi of SirT1 reduced it (**Figure 5C**). Consistent with a stimulatory role for SirT1, cells treated with two different SirT1 specific small molecule activators, SRT1720 and SRT2183, also showed a similar increase in luciferase activity (**Figure 5C**) (Milne et al., 2007).

We were next interested to determine whether this activation had any consequences on osteoblast differentiation. Consistent with a stimulatory role, primary osteoblasts treated with SRT2183 treatment showed a dose-dependent increase in alkaline phosphatase activity and mineralization (**Figure 5D**). Importantly, SRT2183 treatment also resulted in an induction of endogenous Runx2 targets, confirming SirT1 as an important activator of Runx2 activity (**Figure 5E**).

Intriguingly, another small molecule activator of SirT1, resveratrol, has recently been shown to protect against age-related bone loss in mice (Pearson et al., 2008). To determine whether this was in part due to Runx2 activation, we treated mice with 400mg/kg/day of resveratrol for 4 months and analyzed expression of Runx2 targets in whole-calvaria (skullcap). Notably, we observed a similar hyperactivation of Runx2 targets in resveratrol treated mice, validating SirT1 as a physiologically relevant activator of Runx2 (**Figure 5F**). These findings also suggest that the anabolic effects of resveratrol on bone might be mediated through the activation of Runx2 by SirT1.

DISCUSSION

Here, we present evidence that SirT1 is a positive regulator of bone mass. Much like whole-body SirT1 knockouts (WBKO), ObKOs develop an osteoporotic phenotype that is associated with decreased osteoblast numbers. However, unlike the WBKOs, ObKOs have no changes in osteoclast numbers nor display any developmental defects. These data confirm that SirT1 promotes osteoblast differentiation cell autonomously *in vivo*. Interestingly, ObKOs display an osteoporotic phenotype only during adulthood, suggesting the presence of bone remodeling defect. Consistent with this, ObKOs show a compromised ability to make new bone, a common characteristic associated with age-related osteoporosis.

Our data further indicate that the effects of SirT1 on osteoblast differentiation are largely independent of PPAR γ , which SirT1 has previously been shown to repress. Several lines of evidence point to this: 1) PPAR γ expression itself is undetectable in the osteoblast cells; 2) PPAR γ downstream targets are not induced in knockout cells; and 3) previous reports have indicated that PPAR γ is important primarily in osteoblast commitment, and not during differentiation. It is therefore notable that the 2.3kb collagen type I promoter used in this study has been shown to be expressed specifically in cells already committed to the osteoblast lineage and therefore not subject to PPAR γ 's influence. As a result, it would be expected that another molecular mechanism must exist to explain the effects of SirT1 on differentiation.

In line with this, we find that SirT1 interacts with and promotes the transcriptional activity of Runx2. This interaction has important physiological implications: cells lacking SirT1 show decreased differentiation associated with reduced expression of Runx2 targets, while cells treated with SirT1 agonists show an inverse effect. Importantly, one of these targets is Osterix (Osx), a transcription factor previously shown to be both necessary and sufficient for osteoblast

differentiation. Therefore, hypoinduction of *Osx* by *Runx2* in the absence of *Sirt1* stands as a plausible model to explain the observed deficiencies in differentiation.

Interestingly, *Runx2* activity has previously been shown to be regulated by members of class I and II HDACs, albeit in a negative manner (Jensen et al., 2007). *Sirt1* is therefore unique in that it functions to enhance, and not repress, *Runx2* activity. The exact mechanism by which *Sirt1* activates *Runx2* is currently unknown and under investigation, though several lines of evidence point towards its deacetylase activity. First, *ObKO* contain a catalytically dead version of *Sirt1*, which is sufficient for onset of the osteoporotic phenotype. Secondly, *Sirt1* agonists result in activation of *Runx2* that is comparable to overexpression of *Sirt1*. These same agonists also lead to increased differentiation of primary osteoblasts, as well as increased bone mass *in vivo*. Current work is focusing on which lysines of *Runx2* are deacetylated by *Sirt1* and how this impacts activity.

Together, these data suggest that *Sirt1* is an ideal pharmacological target for the development of drugs to treat age-related osteoporosis. Intriguingly, a recent report has shown that treatment with the *Sirt1* agonist, resveratrol, partly protects mice against age-related osteoporosis (Pearson et al., 2008). We show that this protection is in fact associated with an induction of *Runx2* targets, suggesting it is mediated, at least in part, by *Sirt1*'s activation of *Runx2*. It will be interesting to determine how resveratrol affects osteoclast activity as well. Further, it will be particularly interesting to examine the second generation of more potent *Sirt1* activators for their effects on bone (Milne et al., 2007). We have already shown that one of these agonists, SRT2813, leads to increased differentiation of primary osteoblasts associated with increased *Runx2* activity. Given the current lack of reliable anabolic drugs for the treatment of

type II osteoporosis, we optimistically await to see if SirT1 activators prove as efficacious treatments for age-related osteoporosis.

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Table 1. Primer Sequences for Osteoblast Markers

Gene	Sequence
Runx2 type II	<i>forward:</i> TGA GAT TTG TGG GCC GGA <i>reverse:</i> TCT GTG CCT TCT TGG TTC CC
Runx2 type I	<i>forward:</i> ATG CGT ATT CCT GTA GAT CCG AGC <i>reverse:</i> GGT GGT CCG CGA TGA TCT
Osteocalcin	<i>forward:</i> AAG CAG GAG GGC AAT AAG GT <i>reverse:</i> TTT GTA GGC GGT CTT CAA GC
Osterix	<i>forward:</i> GCA AGG CTT CGC ATC TGA AA <i>reverse:</i> AAC TTC TTC TCC CGG GTG TGA
Bone Sialoprotein	<i>forward:</i> CAG GGA GGC AGT GAC TCT TC <i>reverse:</i> AGT GTG GAA AGT GTG GCG TT
Osteopontin	<i>forward:</i> AGC AAG AAA CTC TTC CAA GCA A <i>reverse:</i> GTG AGA TTC GTC AGA TTC ATC CG
Msx2	<i>forward:</i> GGG TCT AAA GCG GAA GTC ACT <i>reverse:</i> GAT GGC GAC CAC TTT CTT GTT
Dlx5	<i>forward:</i> TCT CTA GGA CTG ACG CAA ACA <i>reverse:</i> GTT ACA CGC CAT AGG GTC GC
Dlx3	<i>forward:</i> CAC TGA CCT GGG CTA TTA CAG C <i>reverse:</i> GAG ATT GAA CTG GTG GTG GTA G
AP2	<i>forward:</i> GGG GCC AGG CTT CTA TTC C <i>reverse:</i> GGA GCT GGG TTA GGT ATG GG
PPAR γ	<i>forward:</i> TCG CTG ATG CAC TGC CTA TG <i>reverse:</i> GAG AGG TCC ACA GAG CTG ATT
Lipoprotein Lipase	<i>forward:</i> GGG AGT TTG GCT CCA GAG TTT <i>reverse:</i> TGT GTC TTC AGG GGT CCT TAG
Rpl19	<i>forward:</i> AAG CCT GTG ACT GTC CAT TC <i>reverse:</i> CTT CTT GGA TTC CCG GTA TC

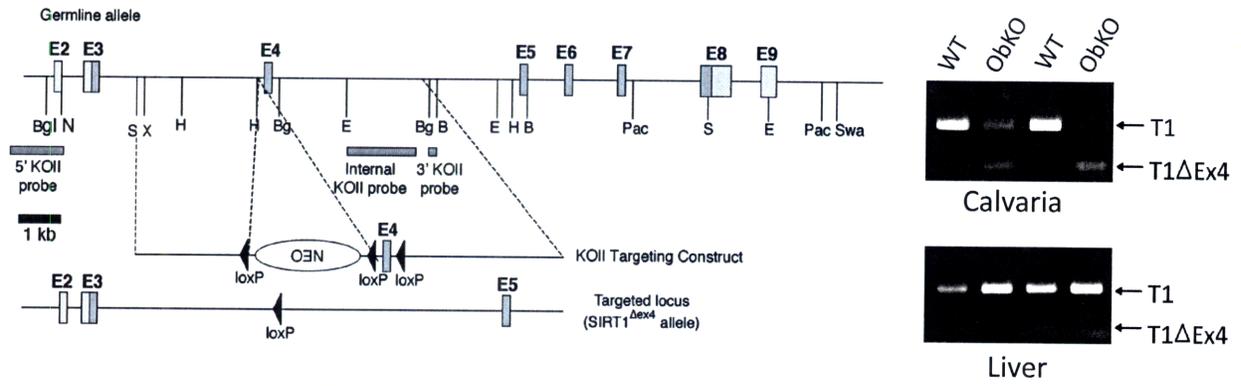
Figure 1. SirT1 osteoblast specific knockouts (ObKOs).

(A) Osteoblast specific deletion of SirT1 was obtained by crossing SirT1 floxed mice with a strain expressing Cre under the 2.3kb Collagen type 1 promoter (Cheng et al., 2003; Liu et al., 2004). Expression of Cre leads to excision of the catalytic domain of SirT1 (exon 4), rendering it functionally dead. RT-PCR using primers flanking exon 4 verifies excision of SirT1 (T1 Δ 4) in bone (neonatal calvaria), but not in other tissues such as liver.

(B) ObKO mice display none of the development defects associated with whole-body knockouts. Error bars represent SEM. ($n \geq 6$ for each group; * $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 1

A



B

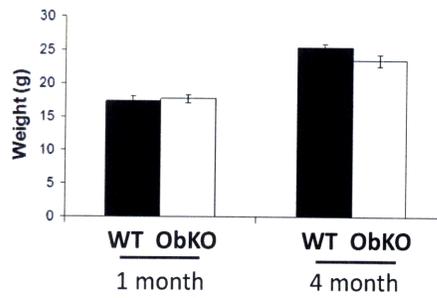


Figure 2. ObKOs display an osteoporotic phenotype associated with decreased osteoblasts.

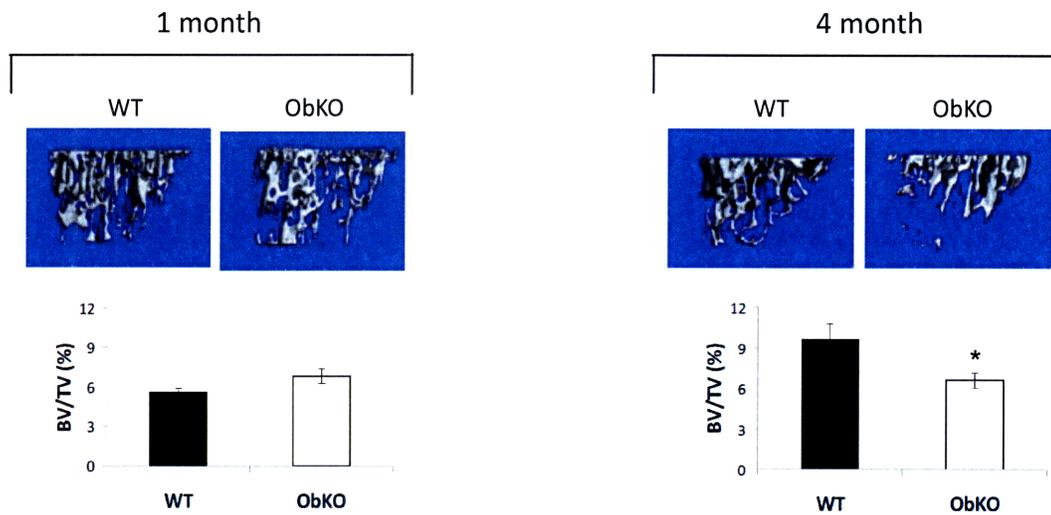
(A) Microcomputed tomography (microCT) shows ObKOs have normal bone mass at 1 month, but display reduced bone volume/total volume (BV/TV) at 4 months of age.

(B) Histology confirms normal bone values at 1 month of age, but reduced bone volume, osteoblast number (Ob S./BS) and bone formation rate (BFR) at 4 months. Osteoclast numbers (Oc S./BS) do not change at either 1 month or 4 months.

Error bars represent SEM. ($n \geq 6$ for each group; * $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 2

A



B

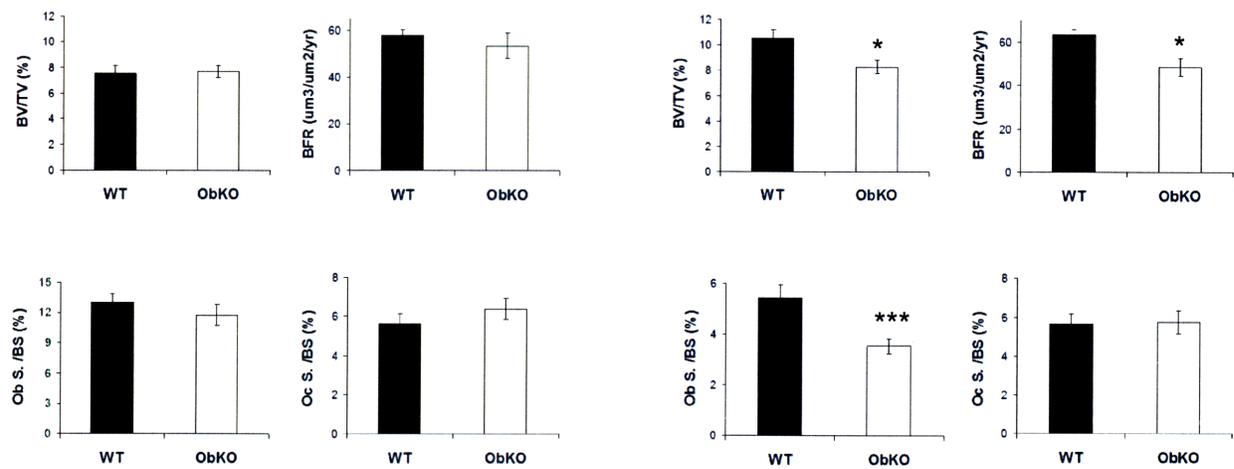


Figure 3. Strategy for *in vitro* osteoblast differentiation.

Primary osteoblasts were isolated from the calvaria of SirT1 flox/flox neonates through serial collagenase/trypsin digestions. Cells were then expanded for a maximum of three passages before being plated for experiments. To minimize any effects on proliferation, cells were infected upon confluency, immediately preceding differentiation, with either vector or Cre adenovirus for 24 hours at 50 MOI. Cell were then allowed to recover for 24 hours before being differentiated with ascorbic acid and β -glycerophosphate.

Figure 3

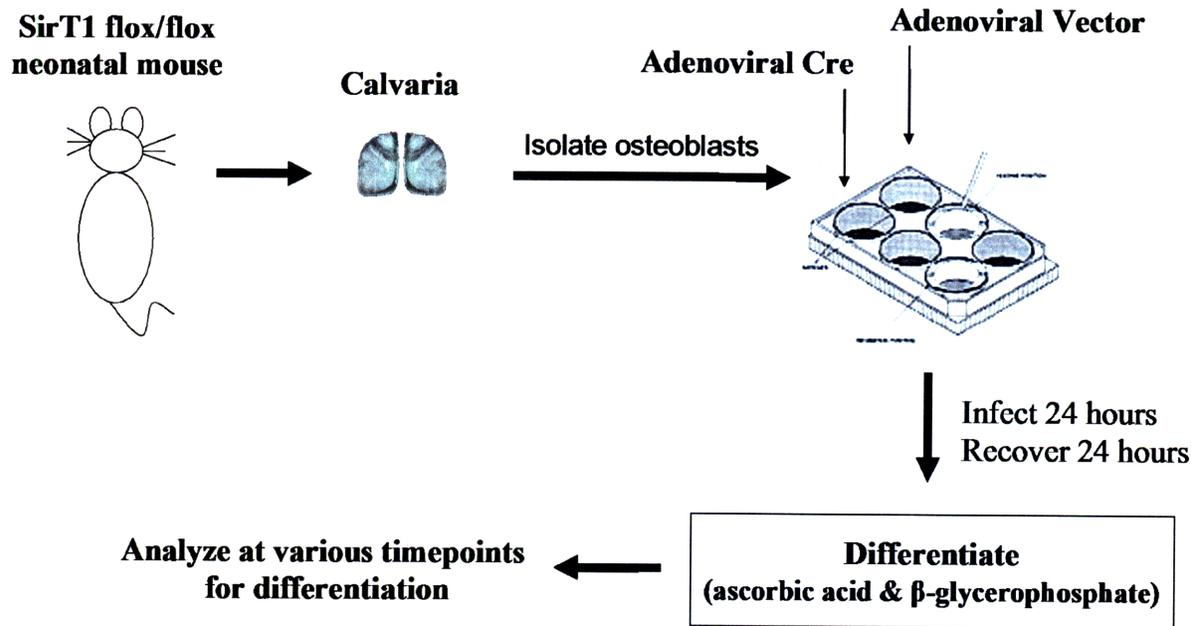


Figure 4. SirT1 deletion reduces differentiation and expression of Runx2 targets.

(A) Primary SirT1 flox/flox osteoblasts were infected with either vector or Cre adenovirus and used for differentiation studies. To minimize any effects on proliferation (as opposed to differentiation), cells infected upon confluency and immediately preceding differentiation. As expected, cells infected with Cre show excision of SirT1 (T1Δ4) as indicated by a smaller PCR product obtained using primers flanking exon 4.

(B) Cre-infected cells show reduced alkaline phosphatase activity, an early marker of osteoblast differentiation.

(C) Cre-infected cells also showed reduced alkaline phosphatase staining and mineralization (as assayed by alizarin red staining).

(D) Q-RTPCR analysis reveals comparable levels of the early osteoblast transcription factors, Msx2, Dlx3, and Dlx5 in both vector and Cre infected cells.

(E) Cre-infected cells express normal levels of Runx2 type II (the predominant isoform found in these cells), but show a near two-fold reduction in the expression of the Runx2 downstream target, Osterix (Osx).

(F) Three other Runx2 targets, Osteocalcin, Osteopontin, and Bone Sialoprotein (BSP), also show reduced expression in Cre infected cells, suggesting a deficiency in Runx2 activity.

Error bars represent SEM. (* p<.05; ** p<.01; *** p<.005).

Figure 4

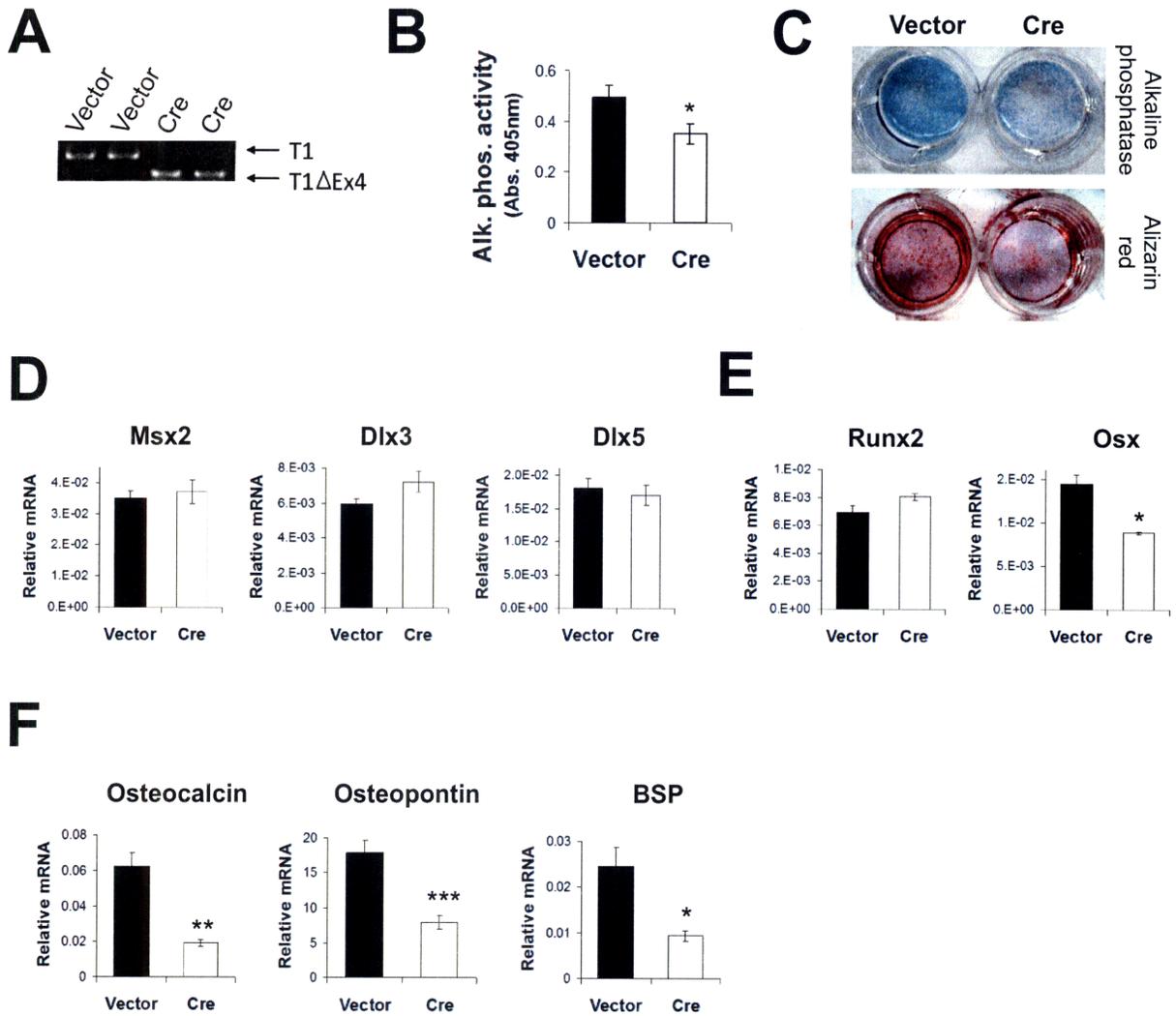


Figure 5. SirT1 interacts with and activates Runx2 transcriptional activity.

(A) Tagged versions of SirT1 and Runx2 interact in 293T cells: Flag-tagged SirT1 is able to co-immunoprecipitate HA-tagged Runx2, and vice versa.

(B) This interaction also exists at the endogenous level. Immunoprecipitation of endogenous Runx2 with two different antibodies (Sigma and Abcam) results in co-immunoprecipitation of SirT1, but not the closely related SirT6 nor the abundantly expressed HSP90 in U2OS osteosarcoma cells.

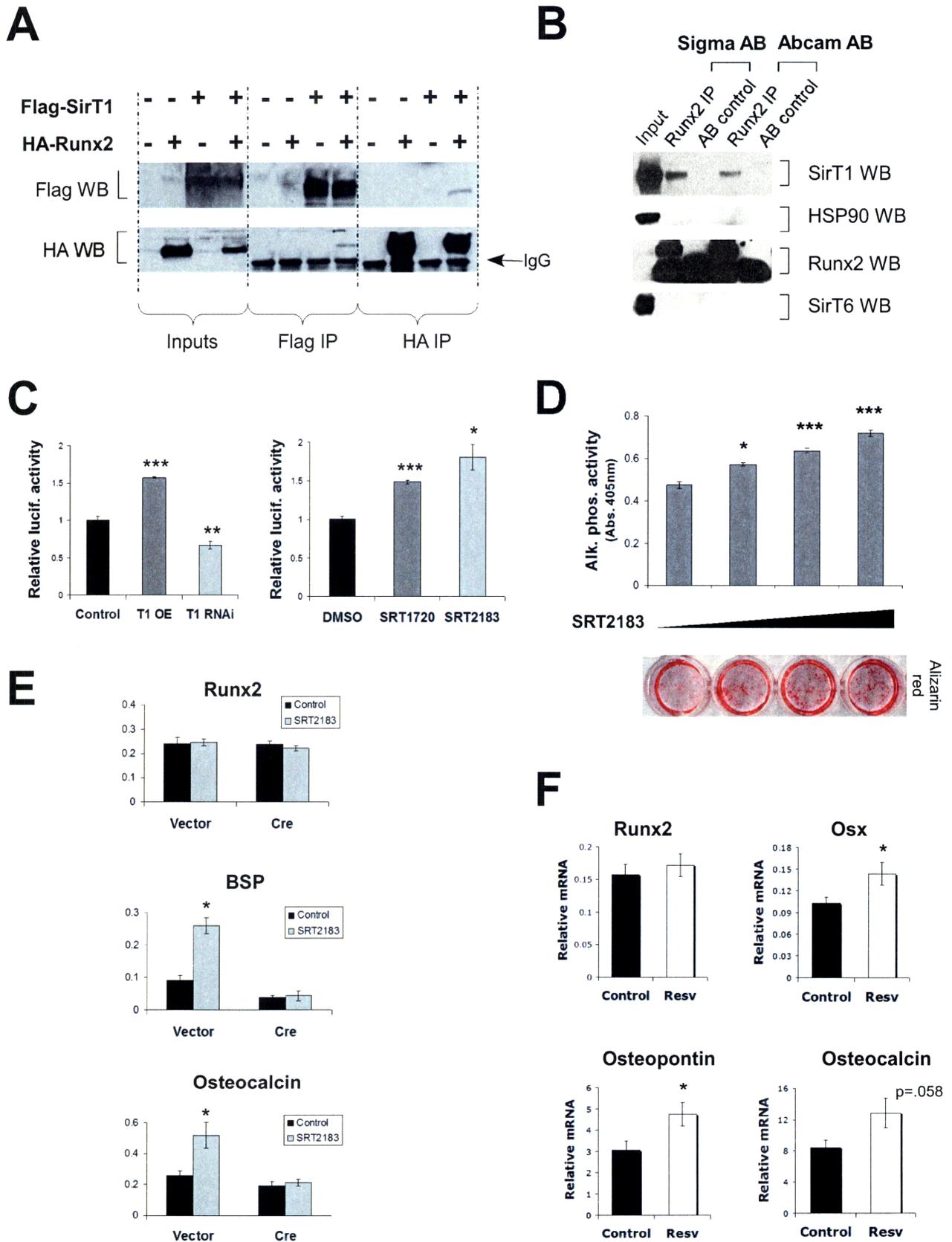
(C) This interaction is accompanied by an increase in Runx2 transcriptional activity as assayed by a Runx2 luciferase reporter (p6OSE2) which contains six Runx2 specific OSE2 elements upstream of luciferase. Overexpression of SirT1 increases luciferase activity, while RNAi of SirT1 decreases it. Further, the SirT1 specific activators, SRT1720 and SRT2183, also result in activation of luciferase that is comparable to overexpression of SirT1 (both used at 1 μ M).

(D) Treatment of primary osteoblasts with SRT2183 during differentiation leads to induction of alkaline phosphatase activity and increased mineralization in a dose-dependent manner (used at .5 μ M, 1 μ M, and 1.5 μ M).

(E) SRT2183 treatment leads to induction of endogenous Runx2 targets in vector but not cre-infected cells, thereby validating SirT1 as an activator of Runx2 in osteoblasts (used at 1 μ M).

(F) Mice treated with 400mg/kg/day of resveratrol, another SirT1 activator, show similar increases in the expression of Runx2 targets in whole calvaria (n=8). These results show that SirT1 is a physiologically relevant activator of Runx2, and provide an explanation for the previously reported anabolic effects of resveratrol on bone (Pearson et al., 2008). Error bars represent SEM. (* p<.05; ** p<.01; *** p<.005).

Figure 5



Chapter 4

SirT1 deletion in osteoclasts causes an osteoporotic phenotype associated with increased osteoclasts and NF κ B hyperactivity

This chapter will be submitted for publication. The authors are Kayvan Zainabadi, James Edwards, Cassie Liu, Gregory Mundy, and Leonard Guarente. The author and James Edwards performed this work as an equally-shared, joint project; all mouse work was performed in the lab of Leonard Guarente, while all bone analyses were performed in the lab of Gregory Mundy. Cell work was divided evenly between the two labs.

SUMMARY

SirT1, the mammalian orthologue of yeast Sir2, has previously been shown to deacetylate and repress NF κ B, a transcription factor important for osteoclast differentiation. To determine the exact role of SirT1 in osteoclastogenesis, we used Cre/Flox technology to create osteoclast specific SirT1 knockout mice (OcKO) using the Lysozyme-M promoter. OcKOs are grossly normal and display none of the developmental defects associated with whole-body SirT1 knockout mice. However, like whole-body knockouts, OcKOs show osteoporosis both at one month and four months of age that is associated with increased osteoclast numbers. As expected, osteoclasts from knockout mice show hyperacetylation of the p65/RelA subunit of NF κ B, and increased expression of a number of NF κ B downstream targets, including the major osteoclast transcription factor, NFATc1. Importantly, the hyperdifferentiation phenotype of knockout cells can be largely rescued through chemical inhibition of NF κ B. These data therefore indicate that SirT1 regulates bone mass *in vivo* by cell autonomously repressing osteoclast differentiation via inhibition of NF κ B.

INTRODUCTION

Bone consists of two principle cell types: the mesenchymal derived osteoblasts that form bone, and the haematopoietic derived osteoclasts that break down bone. Osteoblast and osteoclasts work together during adulthood to continually replace old bone with new bone. This process is termed bone remodeling, and is discrete from bone modeling, which is the patterning of the skeleton during development. Through the coupled action of osteoblasts and osteoclasts, bone remodeling helps maintain the strength and resilience of the skeletal system throughout life (Harada and Rodan, 2003). However, this remodeling process becomes uncoupled with aging, resulting in osteoporosis. This can result either due to upregulation of osteoclast activity (Type I, post-menopausal osteoporosis) or downregulation of osteoblast activity (Type II, age-related osteoporosis).

The differentiation process of osteoclasts is comparatively less complex than that of osteoblasts. Unlike osteoblasts, osteoclasts are haematopoietic cells derived from a monocytic lineage which can differentiate either towards macrophages or osteoclasts (Teitelbaum, 2000). Further, differentiation down either the macrophage or osteoclast lineage does not appear to be mutually exclusive (Väänänen and Laitala-Leinonen, 2008). In fact, osteoclasts are often portrayed as highly specialized macrophages.

Since any misregulation in the differentiation of osteoclasts can potentially lead to osteoporosis, their activity is tightly regulated by their osteoblast counterparts. Osteoblasts promote the differentiation of osteoclasts through two main factors: MCSF (macrophage colony stimulating factor), and RankL (receptor activator of NFκB ligand). These two factors together have been shown to be both sufficient and necessary for osteoclast differentiation (Boyle et al., 2003; Lacey et al., 1998; Kong et al., 1999). While MCSF promotes the survival and

proliferation of the monocyte precursor, RankL specifically initiates the early steps of osteoclast differentiation (Yoshida et al., 1990; Yasuda et al., 1998). Accordingly, MCSF is required for both macrophage *and* osteoclast differentiation, and will differentiate cells to macrophages in the absence of RankL.

Binding of RankL to the Rank receptor leads to a critical step in the osteoclast differentiation program: activation of NF κ B (Asagiri and Takayanagi, 2007). In non-stimulated cells, NF κ B family members reside in the cytoplasm where they are transcriptionally inactive. However, treatment with RankL leads to activation of the Inhibitor of the κ B Signaling Pathway Kinase (IKK), which phosphorylates and thereby promotes the ubiquitin-dependent degradation of the I κ Bs - the proteins responsible for sequestering the NF κ Bs in the cytoplasm. This in turn leads to NF κ B translocation to the nucleus where it activates transcription of target genes (Wong et al., 1998). Underscoring the importance of this cascade, the activation of NF κ B in response to RankL has been shown to be absolutely necessary for the differentiation of osteoclasts (Bar-Shavit, 2007; Asagiri and Takayanagi, 2007).

Once in the nucleus, one of the critical targets of NF κ B is NFATc1 (Nuclear Factor of Activated T-cells). NF κ B has been shown to activate expression of NFATc1 by binding directly to κ B sites in its promoter (Takayanagi, 2007). This induction appears essential for osteoclastogenesis: NF κ B inhibitors that prevent activation of NFATc1 completely block osteoclast differentiation, as do mutations in NFATc1 (Boyle et al., 2003; Takayanagi, 2007). Conversely, NFATc1 overexpression can drive osteoclastogenesis even in the absence of RankL (Asagiri and Takayanagi, 2007). This is in part because NFATc1 directly activates the transcription of most major osteoclast genes, including TRAP (Tartate Resistant Acid Phosphatase), Calcitonin Receptor, and Cathepsin K, as well as of itself (Ishida et al., 2002;

Takayanagi et al., 2002; Boyle et al., 2003). Though the exact molecular details have yet to be worked, the ability of NFATc1 to promote the onset of the osteoclast transcriptional program has earned it the title of a ‘master osteoclast transcription factor’ (Asagiri and Takayanagi, 2007).

Sirt1 has previously been shown to be a repressor of NFκB signaling: Sirt1 deacetylates the RelA/p65 subunit of NFκB at lysine 310 and thereby inhibits its transcriptional activity (Yeung et al., 2004). This repression has been shown to have significant physiological consequences: it sensitizes cancer cells to TNFα-induced apoptosis (Yeung et al., 2004); protects against microglia dependent amyloid-β toxicity (Chen et al., 2005); and lowers activation of pro-inflammatory pathways associated with aging (Pfluger et al., 2008). However, the consequences of this repression in the context of osteoclastogenesis have yet to be explored.

To address this, we have created mice lacking Sirt1 specifically in osteoclasts. These mice develop an osteoporotic phenotype associated with increased osteoclast numbers. As expected, osteoclasts from knockout mice show hyperacetylation of NFκB and enhanced expression of NFκB downstream targets. Importantly, chemical inhibition of NFκB largely rescues the hyperdifferentiation phenotype of knockout cells, thereby functionally implicating NFκB. Our results therefore indicate that Sirt1 regulates bone mass *in vivo* through cell autonomous repression of osteoclast differentiation.

MATERIALS AND METHODS

Animal experimentation

All mice were housed under controlled temperature (25 ±1°C S.D.) and lighting conditions. Food provided was normal chow. Mice were cared for in accordance with the MIT animal care committee.

Microcomputed tomography (micro-CT) and X-ray analysis

Dissected bones were analysed for overall mineral content by X-ray (Faxitron). Tibia and femur were analyzed by micro-CT scanning (uCT40, Scanco) at an isotropic voxel size of 12um (resolution 280). After the growth plate was identified in each scan set, the metaphyseal region 200um below this area was scanned and analysed for alterations in trabecular bone parameters. Also, a 200um region of femoral diaphysis in the exact center of each bone was calculated, scanned and assessed for alterations in cortical bone dimensions.

Histologic and histomorphometric analyses

Lumbar vertebrae and long bones were collected following sacrifice and fixed for up to 48 hours in 10% formalin (Fisher). Undecalcified regions of vertebrae were processed and embedded in a methacrylate-based resin (Sigma) and sectioned at 5µm. Sections were deplastized and stained for bound calcium ions using the Von Kossa procedure with a van Gieson counterstain, or using a post-coupling staining technique for tartrate-resistant acid phosphatase. Bone volume and cellular distribution was quantified histomorphometrically using Osteomeasure quantification software (Osteometrics). Osteoblasts and osteoclasts were identified based on both morphology and staining with alkaline phosphatase or tartrate resistant acid phosphatase (TRAP), respectively. Bone formation rate was determined by double-labeling with calcein injection at 4-day intervals, with animals sacrificed on the sixth day.

Differentiation of primary osteoclasts

Tibia and femurs from adult mice (3 – 6 months) were isolated and flushed with α -MEM with a 25G needle. Cells were then resuspended by pipetting 10 times with a 2ml pipette, filtered through a 70µm nylon filter (BD Falcon), spun down at 1,000rpm and resuspended with fresh α -MEM. A small aliquot of the resulting cells was then incubated in 5% acetic acid for 1' to lyse red blood cells and counted under a hemacytometer. Cells were then plated at approximately 1

million/cm² in α -MEM overnight, which was changed to differentiation media the following morning. For macrophages differentiation, MCSF (50ng/ml) alone was used; for osteoclast differentiation, MCSF (50ng/ml) and RankL (30-50ng/ml) was used.

TRAP and crystal violet staining

Once cells had differentiated, cells were fixed with 10% neutral buffered formalin for 10' and then washed 2x with water. Macrophages were stained for total colonies with 0.05% crystal violet dissolved in water for 30' and then washed extensively. Osteoclasts were stained using the TRAP Leukocyte Alkaline Phosphatase Kit (Sigma) according to manufacturer's instructions. Multinucleated TRAP+ cells were counted as osteoclasts.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIZOL (Invitrogen) and cleaned-up with the RNeasy MinElute Cleanup kit (Qiagen). 1 ug of cDNA was synthesized from total RNA by first-strand synthesis with random hexamers using SuperScript III reverse transcriptase (Invitrogen). cDNA was then subjected to realtime-PCR analysis with gene-specific primers in the presence of iQ SYBR green (Bio-Rad) (**Table 1**). Depending on the abundance of target transcript, stock cDNA was diluted anywhere in the range of 1:3 to 1:20 to achieve optimal crossing point (CP) values. Relative mRNA abundance was obtained by normalization to Rpl19 or GAPDH levels. The protocol for qRT-PCR is as follows: the cDNA was denatured by heating the reaction to 95°C for 5', then 45 cycles at 95°C for 10", 55°C for 15", and 72°C for 25". After amplification, a melting curve analysis was taken to ensure the presence of only one amplification product.

Statistical analysis

Analysis was performed using an unpaired Student's t-test, with p-values less than 0.05 considered significant. All data is presented \pm standard error of the mean (SEM).

RESULTS

Generation of osteoclast specific SirT1 knockout (OcKO) mice

In Chapter 2, we demonstrated that whole-body SirT1 knockout mice display an early osteoporotic phenotype that is, in part, due to increased osteoclast numbers. To gain further insight into the role of SirT1 in osteoclast differentiation, we generated osteoclast specific knockouts by crossing SirT1 floxed mice with Lysozyme-M Cre mice. Previous studies have shown that Lysozyme-M is expressed specifically in the monocyte precursors that give rise to osteoclasts and macrophages (Clausen et al., 1999; Kenner et al., 2004). Much like the previously mentioned osteoblast knockouts, OcKOs appear grossly normal and are indistinguishable from their wildtype littermates (**Figure 1A**).

OcKO mice show an osteoporotic phenotype associated with increased osteoclast numbers

Microcomputed tomography (micro-CT) reveals OcKOs have reduced bone mass at both 1 month and 4 months of age, similar to whole-body SirT1 knockouts (**Figure 1B**). Histological examination confirms a reduction in the amount of mineralized bone in both 1 and 4 month old OcKOs (**Figure 1C**). Importantly, the osteoporotic phenotype is associated with an increase in osteoclast numbers; osteoblast numbers and bone formation rate are not affected (**Figure 1C**). Since OcKOs are missing SirT1 specifically in their osteoclasts, these results indicate that SirT1 cell autonomously represses osteoclast differentiation *in vivo*.

SirT1 represses osteoclast differentiation via inhibition of NF κ B

To gain further insight into how SirT1 affects osteoclastogenesis, we performed *in vitro* osteoclast differentiation assays (**Figure 2A**). Cells from wildtype or knockout mice were differentiated towards osteoclasts with MCSF and RankL, or macrophages with MCSF alone, as a control. Interestingly, knockout cells show a marked increase in differentiation towards the osteoclast lineage, but not towards macrophages (**Figure 2B**). This suggests that SirT1 specifically represses osteoclast differentiation. In line with this, microscopic examination shows that knockout cells give rise to a larger number of large multinucleated TRAP positive cells that are hallmarks of osteoclasts (**Figure 2C**). Further, treatment with even a low dose of RankL is sufficient to induce osteoclastogenesis in knockout cells, suggesting a hypersensitivity to RankL signaling.

SirT1 is a known repressor of NFκB via deacetylation of p65/RelA. Since NFκB activation is one of the major downstream targets of the RankL, we hypothesized that NFκB hyperactivation might explain the observed increases in differentiation. Consistent with this, we find hyperinduction of a number of osteoclast genes in knockout cells, including those which are downstream targets of NFκB (**Figure 3A**). Further, knockout cells show hyperacetylation of the p65 subunit of NFκB at lysine 310, which SirT1 normally deacetylates (**Figure 3B**). To determine whether NFκB hyperactivation sensitizes knockout cells to RankL, we treated cells with a short burst (24 hours) of RankL and then measured expression of the major NFκB target, NFATc1. Consistent with a role for NFκB, knockout cells show a near two fold higher induction of NFATc1, but not that of NFATc2 (a non-NFκB target) (**Figure 3C**). Importantly, chemical inhibition of NFκB with a low dose of BMS 345541 (BMS) largely rescues the hyperdifferentiation phenotype of knockout cells, further implicating NFκB (**Figure 3D**). These

results therefore indicate that SirT1 represses osteoclast differentiation through inhibition of NF κ B.

DISCUSSION

Post-menopausal osteoporosis is associated with increased osteoclast numbers owing to decreased estrogen levels, a natural inhibitor of osteoclastogenesis. While estrogen replacement therapy (ERT) initially appeared as a promising therapy, ERT is now considered too risky since it increases the risk of cardiovascular disease and certain forms of cancer. Further, other therapies, such as the bisphosphonates, act by inhibiting bone resorption, but not the underlying imbalance in osteoclastogenesis. Consequently, these therapies have the unwanted side-effect of largely shutting down bone remodeling, an essential process for bone strength and repair (Harada and Rodan, 2003). As a result, there is a growing concern about the long term use of these drugs for the treatment of post-menopausal osteoporosis. These concerns have grown recently with reports demonstrating higher rates of osteonecrosis (bone death) in women treated with bisphosphonates (Sarasquete et al., 2009). Therefore, there is currently a need for treatments that specifically address the increase in osteoclast numbers associated with menopause. In this regard, genes that regulate osteoclast differentiation are ideal pharmacological targets for the development of novel therapies.

Here, we show that SirT1 is an endogenous inhibitor of osteoclast differentiation. SirT1 performs this function by deacetylating and thereby repressing the p65/RelA subunit of NF κ B (Yeung et al., 2004). In support of this, we find hyperactivation of endogenous NF κ B targets in knockout cells. Interestingly, one of these targets is NFATc1, a transcription factor that has previously been shown to be both necessary and sufficient for osteoclast differentiation (Takayanagi et al., 2002). It is therefore likely that hyperinduction of NFATc1 by NF κ B, in the

absence of SirT1, is a likely model to explain the increased differentiation. Consistent with this, differentiation of knockout cells can be brought to near wildtype levels by the administration of an NFκB inhibitor, functionally implicating NFκB as the key mediator of these effects.

Although the repression of NFκB by SirT1 has been shown to be important in a number of different physiological contexts, this is the first instance implicating it in osteoclastogenesis. Consistent with this, mice lacking SirT1 specifically in their osteoclasts develop an osteoporotic phenotype due to increased osteoclast numbers. These results therefore confirm SirT1 is an *in vivo* regulator of bone mass through cell autonomous repression of osteoclast differentiation. Interestingly, the phenotype of the OcKOs closely mirrors what is commonly seen in post-menopausal osteoporosis.

Given this, it will be interesting to determine how SirT1 agonists affect osteoclast differentiation, and whether these compounds serve as useful therapies for post-menopausal osteoporosis. These studies have thus far been hampered by the fact that resveratrol, a SirT1 agonist, is also a super-agonist for the estrogen receptor (Gehm et al., 1997). However, the recent advent of the second generation of structurally distinct SirT1 agonists should help resolve this complication (Milne et al., 2007). Our results indicate that activation of SirT1 would be predicted to repress osteoclast differentiation, and thereby possibly correct the imbalance in osteoclasts seen in post-menopausal osteoporosis. Further, given the results in Chapter 3, we would predict that these agonists might also enhance new bone formation by stimulating osteoblast activity. Therefore, SirT1 activators might serve as a one-two punch against osteoporosis, by temporarily favoring bone formation over resorption, thereby leading to increased bone mass.

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Table 1. Primer Sequences for Osteoclast Markers

Gene	Sequence
NFATc1	<i>forward:</i> GAC CCG GAG TTC GAC TTC G <i>reverse:</i> TGA CAC TAG GGG ACA CAT AAC TG
NFATc2	<i>forward:</i> CTC GGC CTT TGC CCA TCT C <i>reverse:</i> AGG AGC ACG GAG CAT CTG A
TNF α	<i>forward:</i> CCC TCA CAC TCA GAT CAT CTT CT <i>reverse:</i> GCT ACG ACG TGG GCT ACA G
Interleukin 1	<i>forward:</i> GCA ACT GTT CCT GAA CTC AAC T <i>reverse:</i> ATC TTT TGG GGT CCG TCA ACT
TRAP	<i>forward:</i> CAC TCC CAC CCT GAG ATT TGT <i>reverse:</i> CAT CGT CTG CAC GGT TCT G
rpl19	<i>forward:</i> AAG CCT GTG ACT GTC CAT TC <i>reverse:</i> CTT CTT GGA TTC CCG GTA TC

Figure 1. SirT1 osteoclast specific knockout mice (OcKO) display an osteoporotic phenotype associated with increased osteoclasts.

(A) Osteoclast specific deletion was obtained by crossing SirT1 floxed mice with a strain expressing Cre under the Lysozyme-M promoter (Cheng et al., 2003; Clausen et al., 1999). Expression of Cre leads to excision of the catalytic domain of SirT1 (exon 4), rendering it functionally dead. RT-PCR using primers flanking exon 4 verifies excision of SirT1 (T1 Δ 4) in osteoclasts, but not in other tissues such as liver. OcKO mice display none of the development defects associated with whole-body knockouts.

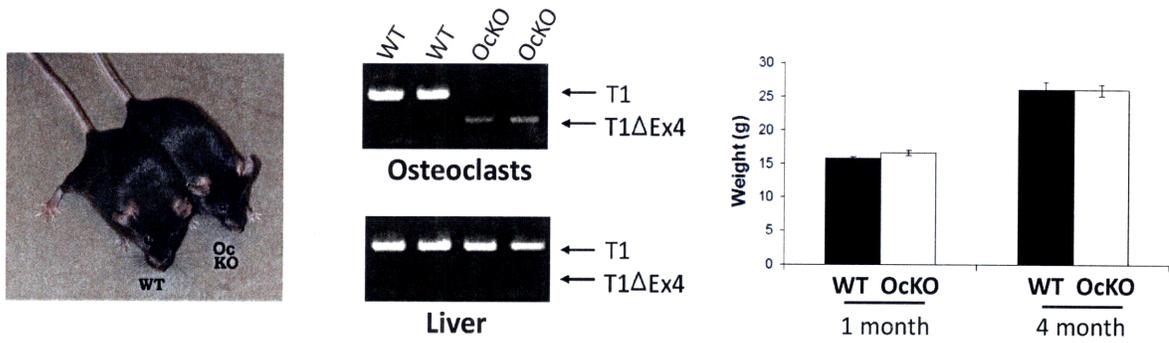
(B) Microcomputed tomography (micro-CT) shows OcKOs have reduced bone volume/total volume (BV/TV) at both 1 month and 4 months of age.

(C) Histology indicates that the osteoporotic phenotype is associated with increased osteoclast number (Oc S./BS) at both ages. Osteoblast numbers (Ob S./BS) and bone formation rate (BFR) are not affected at either age.

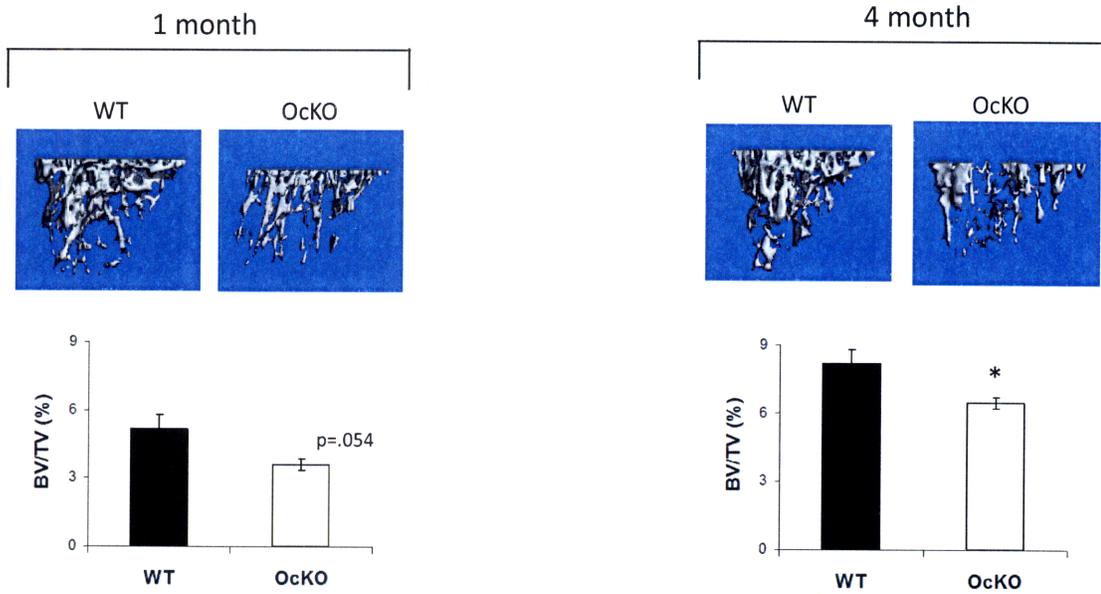
Error bars represent SEM. (n \geq 6 for each group; * p<.05; ** p<.01; *** p<.005).

Figure 1

A



B



C

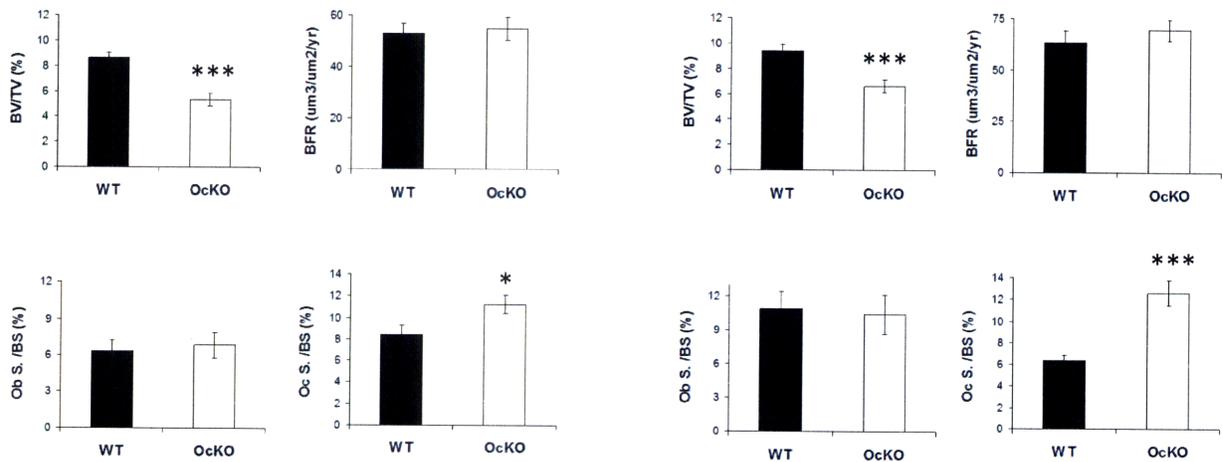


Figure 2. SirT1 represses osteoclast differentiation *in vitro*.

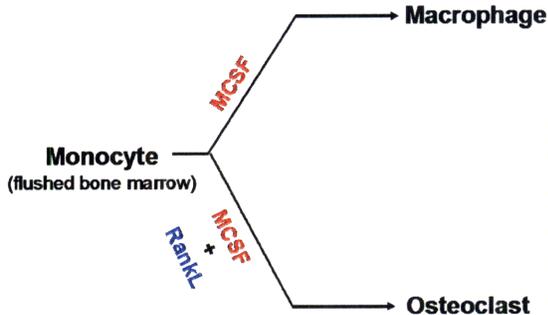
(A) Monocytes from the bone marrow can be differentiated towards either a macrophage lineage (with addition of MCSF), or towards an osteoclast lineage (with addition of MCSF and RankL).

(B) While knockout cells gives rise to equivalent numbers of macrophages (upper), as determined by crystal violet staining, they show an increase in osteoclast formation (lower), as determined by TRAP staining.

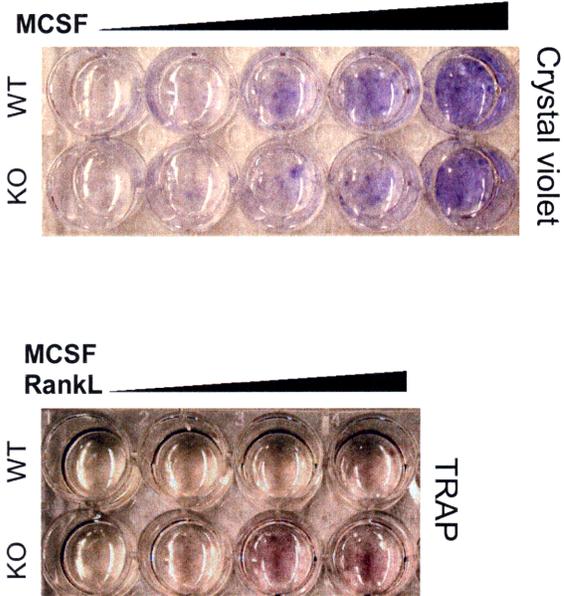
(C) Microscopic examination reveals knockout cells give rise to more multinucleated, TRAP⁺ cells that are hallmarks of osteoclasts at various doses of RankL. Error bars represent SEM. (* p<.05; ** p<.01; *** p<.005).

Figure 2

A



B



C

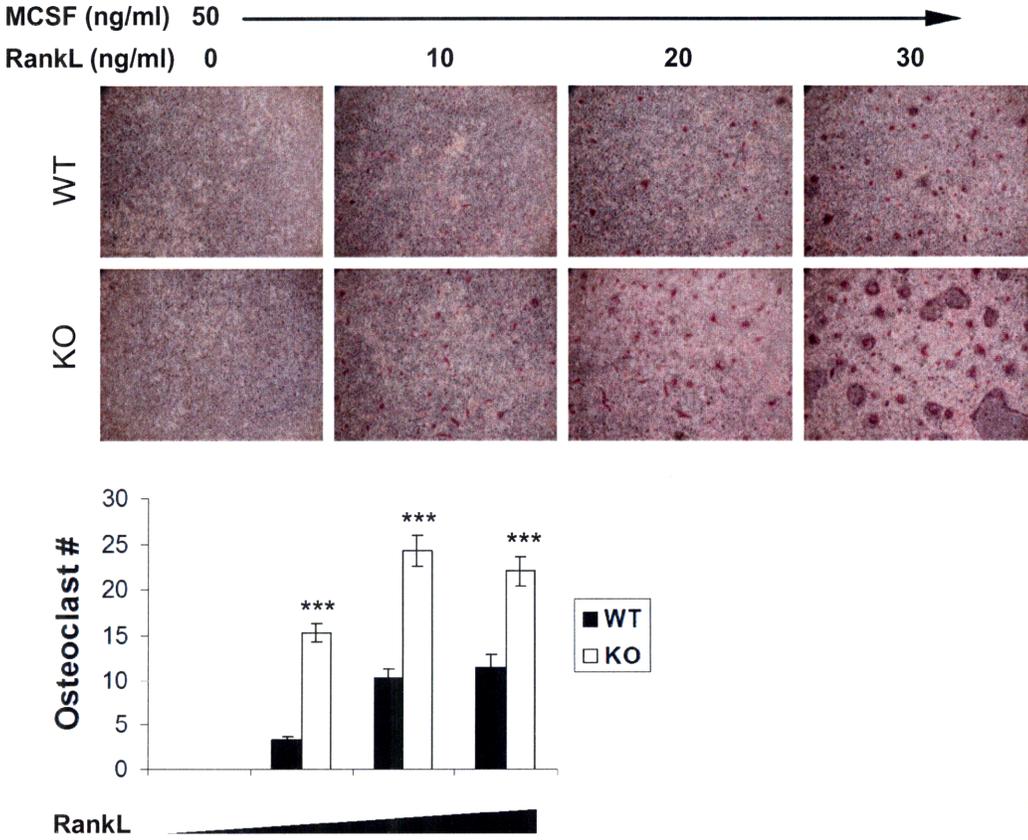


Figure 3. SirT1 represses osteoclastogenesis via inhibition of NFκB.

(A) Knockout cells show increased expression of osteoclast genes (Tartrate Resistant Acid Phosphatase - TRAP), as well as downstream targets of NFκB (Interleukin-1 and Tumor Necrosis Alpha - TNF-alpha).

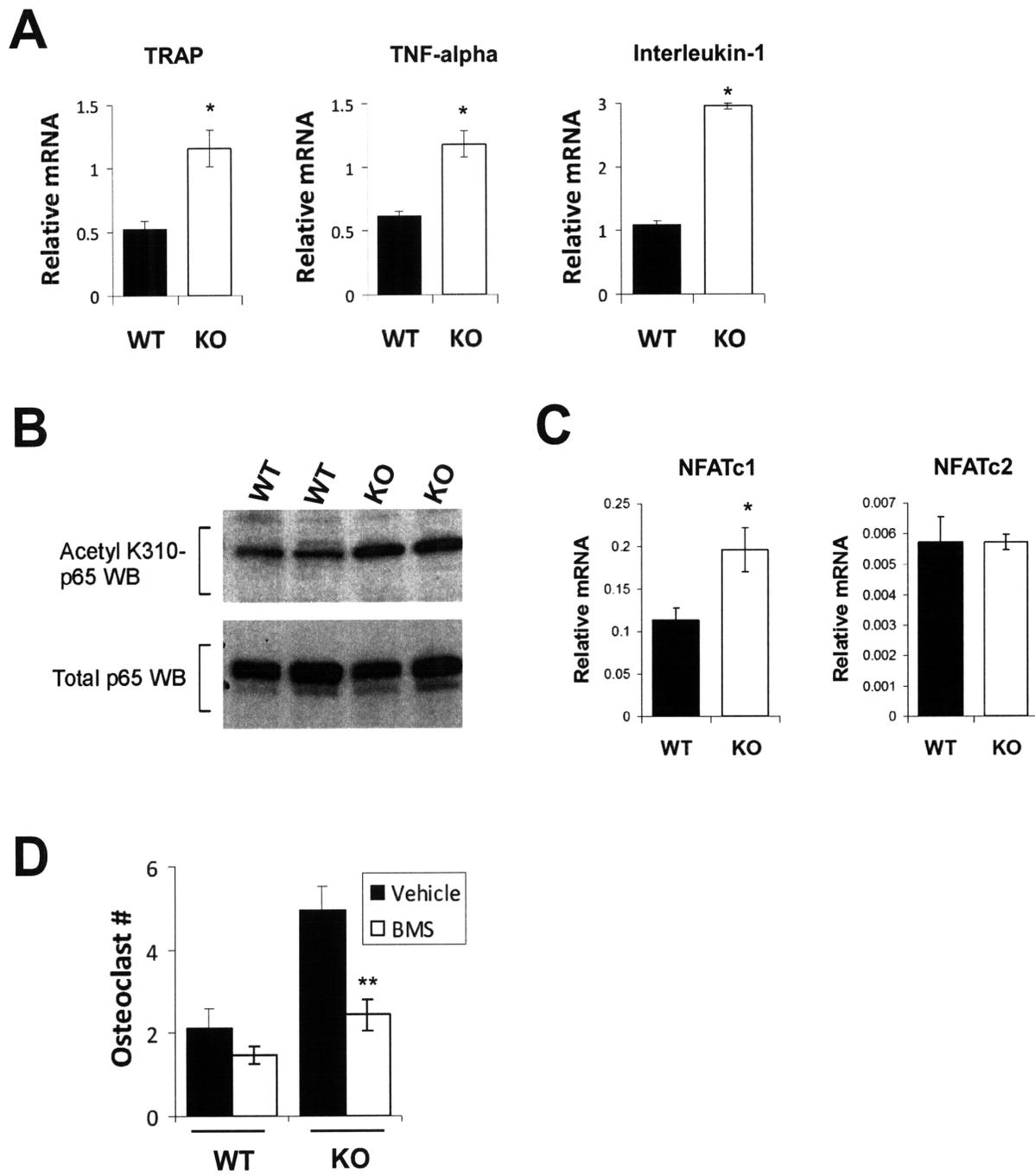
(B) Knockout cells show hyperacetylation of the p65 subunit of NFκB at lysine 310, which has previously been shown to be deacetylated and repressed by SirT1.

(C) To determine if knockout cells were more sensitive to RankL, osteoclast precursors were treated with RankL for 24 hours and then assayed for the expression of the major NFκB target, NFATc1. Consistent with a role for NFκB, knockout cells show hyperactivation of NFATc1, but that of the NFATc2 (a non-NFκB target).

(D) Chemical inhibition of NFκB with a low dose of BMS 345541 (BMS) rescues the hyperdifferentiation phenotype of knockout cells, functionally implicating NFκB.

Error bars represent SEM. (* p<.05; ** p<.01; *** p<.005).

Figure 3



Chapter 5

Calorie restriction results in increased bone mass, possibly through a SirT1 dependent mechanism

This chapter will be submitted for publication. The authors are Kayvan Zainabadi, James Edwards, Cassie Liu, Gregory Mundy, and Leonard Guarente. The author and James Edwards performed this work as an equally-shared, joint project; all mouse work was performed in the lab of Leonard Guarente, while all bone analyses were performed in the lab of Gregory Mundy.

SUMMARY

Calorie restriction (CR) extends the lifespan of virtually every organism tested. In mammals, CR forestalls many of the diseases associated with aging, though its role in osteoporosis remains unclear. To address this, we analyzed mice that were placed under eight months of CR and found a striking increase in bone mass. This increase was associated with an increase in osteoblast and a decrease in osteoclast numbers, a phenotype that is the mirror opposite of SirT1 knockout mice. Linking SirT1 with CR, we observe a two-fold induction of SirT1 expression in the bones of CR mice, as well as an increase in NAD/NADH levels. To determine if SirT1 had a role in mediating these effects, we performed CR on SirT1 whole-body knockout mice. Intriguingly, SirT1 knockout mice fail to show an increase in bone mass upon CR, suggesting SirT1 is required these effects.

INTRODUCTION

It has been known for over 70 years that moderate reductions in food intake can extend the lifespan of animals. This regimen, termed calorie restriction (CR), has since been shown to extend the lifespan of virtually all organisms, including yeast, worms, flies, rodents and likely primates (Koubova and Guarente, 2003). The fact that CR works in such widely different organisms suggests that it has been selected for early in evolution, possibly as a survival mechanism during times of food scarcity.

In mammals, CR protects against many diseases associated with aging. For instance, mice fed a diet 30-40% below ad libitum (AL) levels are more resistant to the development of cardiovascular disease, diabetes, autoimmune disease, kidney disease, as well as a number of neurodegenerative and neoplastic disorders (Koubova and Guarente, 2003). In addition, CR also leads to a healthy metabolic profile associated with longevity, including lower blood glucose levels, increased insulin sensitivity, and reduced inflammatory cytokines. Other well documented physiological changes are reduced fertility, lower core body temperature, and changes in the neuroendocrine system including lower levels of growth hormone, thyroid stimulating hormone, IGF-1, gonadotropins; and increased glucocorticoids, catecholamines and glucagons. Importantly, these numerous changes are associated with a highly robust extension in both average and maximal lifespan of animals. This begs the question: how exactly does CR promote longevity?

Evidence has emerged that the Sir2 family of NAD-dependent deacetylases might be important mediators of CR (Guarente and Picard, 2005). The enzymatic function of Sir2 appears aptly suited for this function: Sir2 proteins are members of the novel class III HDACs (histone deacetylases) which use NAD (instead of water) as a co-factor in the deacetylation of lysine

residues (Imai et al., 2000). Since NAD is a critical intermediate in metabolism, Sir2 activity is thought to be regulated by the energy status of the organism (Guarente and Picard, 2005). This has in fact been shown to be the case: in yeast CR leads to an increase in the NAD/NADH ratio which activates Sir2 silencing activity (Lin et al., 2004). Further, Sir2 appears indispensable for CR-induced longevity: yeast carrying mutations in Sir2 do not live longer upon CR (Lin et al., 2000). Moreover, Sir2 overexpression (or activation with the small molecule activator, resveratrol) extends lifespan of multiple organisms in a manner resembling CR (Howitz et al., 2003; Tissenbaum et al., 2001; Rogina and Helfand, 2004; Wang and Tissenbaum, 2006). In line with this, Sir2 hyperactivation does not further extend the lifespan of animals already on CR, suggesting Sir2 and CR are in the same pathway (Lin et al., 2000; Howitz et al., 2003).

Recent evidence suggests that a similar mechanism might exist in mammals. CR results in an increase in the NAD/NADH ratio, as well as expression of SirT1, in multiple tissues (Cohen et al., 2004; Nisoli et al., 2005; Chen et al., 2008). Further, SirT1 knockout mice fail to respond to several physiological aspects of CR, including increased activity, upregulation of metabolism and increased lifespan (Cohen et al., 2004; Chen et al., 2005; Boily et al., 2008). Additionally, mice treated with resveratrol show a transcriptional profile that closely resembles that of CR (Baur et al., 2006). Intriguingly, resveratrol has recently been shown to protect mice against age-related osteoporosis (Pearson et al., 2008).

Although CR has been shown to be protective against numerous age-related diseases, its role in bone health has so far been inconclusive (Tatsumi et al., 2008; Kalu et al., 1984; Sanderson et al., 1997). For instance, it has been reported that CR either increases, decreases or has no effect on bone mass depending on the specifics of the study, such as the type of CR used (30-40% daily food restriction or every other day feeding); whether mice, rats, or primates are

used; and what age CR is initiated at and for how long. These disparate findings therefore likely reflect the lack of a standardized CR protocol, which is particularly troubling since insufficiency of calcium and phosphate levels in some CR diets have been reported to cause bone loss (Redman et al., 2008). It has also previously been reported that if CR is initiated too early, it can impede the natural maturation of the skeleton, resulting in osteopenia (low peak bone mass) (Gat-Yablonski et al., 2004). A recent study has attempted to bridge these disparate findings by showing that life-long CR in mice in fact has biphasic effects on bone metabolism – short term CR reduces bone mass whereas prolonged CR protects bone against osteoporosis (Tatsumi et al., 2008). However, there is currently no consensus on the effects of CR on bone.

To resolve this, we have obtained CR mice from a standard source (the National Institutes of Aging). These mice, which have been on CR for 8 months, show significant increases in bone mass that is associated with decreased osteoclast and increased osteoblast numbers. Interestingly, whole-body SirT1 knockout mice placed on a similar CR diet fail to show an increase in bone mass, suggesting SirT1 is required to mediate these effects.

MATERIALS AND METHODS

Animal experimentation

All mice were housed under controlled temperature ($25 \pm 1^\circ\text{C}$ S.D.) and lighting conditions. Food provided was normal chow. Mice were cared for in accordance with the MIT animal care committee.

CR experiments

Mice were obtained from the National Institutes of Aging that were 12 months old and had been on a 60% ad libitum diet starting at 4 months of age. Once received, mice were kept on CR for an additional 2 weeks in order to re-equilibrate them to the new surroundings. In CR

experiments performed at MIT, the amount of food eaten by each group was measured over a week and averaged (ad libitum). This amount was reduced by 35% gradually over a 2 week span for the CR group. CR Mice were then kept on the 65% ad libitum diet for 4 months and weighed weekly. As reported in Chen et al., 2005, we noted an increase in activity in wildtype, but not knockout mice.

Microcomputed tomography (micro-CT) and X-ray analysis

Dissected bones were analyzed for overall mineral content by X-ray (Faxitron). Tibia and femur were analyzed by micro-CT scanning (uCT40, Scanco) at an isotropic voxel size of 12µm (resolution 280). After the growth plate was identified in each scan set, the metaphyseal region 200µm below this area was scanned and analyzed for alterations in trabecular bone parameters. Also, a 200µm region of femoral diaphysis in the exact center of each bone was calculated, scanned and assessed for alterations in cortical bone dimensions.

Histologic and histomorphometric analyses

Lumbar vertebrae and long bones were collected following sacrifice and fixed for up to 48 hours in 10% formalin (Fisher). Undecalcified regions of vertebrae were processed and embedded in a methylmethacrylate-based resin (Sigma) and sectioned at 5µm. Sections were deplastasized and stained for bound calcium ions using the Von Kossa procedure with a van Gieson counterstain, or using a post-coupling staining technique for tartrate-resistant acid phosphatase. Bone volume and cellular distribution was quantified histomorphometrically using Osteomeasure quantification software (Osteometrics). Osteoblasts and osteoclasts were identified based on both morphology and staining with alkaline phosphatase or tartrate resistant acid phosphatase (TRAP), respectively.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cells or tissues using TRIZOL (Invitrogen) and cleaned-up with the RNeasy MinElute Cleanup kit (Qiagen). For calvaria preps, non-osseous tissue was first removed in ice-cold PBS, then calvaria was minced in Trizol, and homogenized using a Tissue Tearor homogenizer (VWR). Lysates were then spun down at 15,000g for 10minutes, with the resulting supernatant used for RNA isolation according to manufacturer's instructions.

1 ug of cDNA was synthesized from total RNA by first-strand synthesis with random hexamers using SuperScript III reverse transcriptase (Invitrogen). cDNA was then subjected to realtime-PCR analysis with gene-specific primers in the presence of iQ SYBR green (Bio-Rad). Relative mRNA abundance was obtained by normalization to Rpl19 levels.

NAD/NADH measurements

NAD and NADH measurements from whole calvaria were made using the Fluoro NAD kit (Cell Technology) according to manufacturer's instructions. Values were normalized to total protein content.

Statistical analysis

Analysis was performed using an unpaired Student's t-test, with p-values less than 0.05 considered significant. All data is presented \pm standard error of the mean (SEM).

RESULTS

Calorie restriction (CR) results in increased bone mass associated with increased osteoblast and decreased osteoclast numbers

Calorie restriction has been shown to forestall many diseases of aging, though its effects on bone have been equivocal. In an attempt to resolve this, we analyzed CR mice obtained from the central colony maintained at the National Institutes of Aging (NIA). The mice (C57BL/6) used in this study were 12 months old and had been placed on a CR diet consisting of 60% ad

libitum (AL) levels for 8 months (starting at 4 months of age). As expected, mice on the CR diet are leaner and show a significant reduction in overall body weight (**Figure 1A**). Micro-CT scans reveal a clear increase in the bone mass of CR mice, including a 32% increase in bone mineral density (BMD) and a 38% increase in bone volume/total volume (BV/TV) (**Figure 1B**). These results indicate CR has a positive role on bone mass.

To determine how CR leads to these increases, we performed histology on the L3-L4 vertebrae of ad libitum and CR mice. Gross histology confirms a clear increase in the amount of mineralized bone in the vertebrae of CR animals (**Figure 2A,B**). Cellular counts reveal that this increase is associated with a decrease in osteoclast and an increase in osteoblast numbers per bone surface (**Figure 2B**). Intriguingly, these results are the mirror opposite of what is observed in whole-body SirT1 knockout mice.

CR increases bone mass possibly through a SirT1 dependent mechanism

Given the correlation between CR, SirT1 and bone mass, we were next interested to determine whether any of these effects were mediated through SirT1. CR has previously been shown to lead to increases in the NAD/NADH ratio, as well as expression of SirT1, in multiple mammalian tissues (Cohen et al., 2004; Nisoli et al., 2005; Chen et al., 2008). Consistent with this, we find a two fold induction of SirT1 expression, and a trend towards increased NAD/NADH levels, in the calvaria of CR mice (**Figure 3A,B**). These results suggest that SirT1 activity is induced during CR in bone.

To determine whether SirT1 is required for the observed effects of CR, we placed a cohort of 12 month old wildtype or whole-body knockout mice on CR (35% below ad lib levels) for 4 months. As expected, both wildtype and knockout mice show significant weight loss on the CR diet (**Figure 4A,B**). Micro-CT analysis shows that at this late age (16 months), both ad lib

fed wildtype and knockout mice show low bone mass, indicating they are both likely osteoporotic (**Figure 4C**). However, even after 4 months of CR, wildtype mice show a near-significant increase in bone mass that mirrors the results obtained with the original NIA mice. However, unlike their wildtype counterparts, knockout mice completely fail to show an increase in bone mass upon CR, suggesting SirT1 might be required for these effects.

DISCUSSION

While CR has been shown to protect against many diseases of aging, its role in osteoporosis has so far been inconclusive. This is likely a result of a number of factors, including the lack of a standardized CR protocol used in previous studies. This is particularly important because if CR is initiated too early in development, it can impede the natural maturation of the skeleton (Gat-Yablonski et al., 2004). This can result in a lower peak bone mass which can be misconstrued as osteoporosis.

Here we show that mice in which CR was initiated during adulthood (4 months of age), and maintained for 8 months, show a marked increase in bone mass. At first glance, this appears as a rather peculiar finding - mice on a CR diet generally enter a catabolic state, resulting in loss of significant body weight. However, it is important to note that not all tissues shrink during CR: for example, while the liver and white adipose tissue become dramatically smaller, the brain and heart remain the same (Koubova and Guarente, 2003). These results, therefore, indicate that CR might in fact be a regulated response, acting differently in various tissues, in order to meet the needs of a low calorie diet.

We surmise that this is in fact the case with bone. We find that CR increases bone mass by specifically altering the balance of osteoblasts and osteoclasts, indicative of a regulated response. How does CR achieve this? We surmised that SirT1 might serve as an ideal candidate

for mediating these effects for several reasons. First, we observe an induction of SirT1 expression in the calvaria of CR mice, as well as an increase in NAD/NADH levels. This is consistent with what has been reported for SirT1 in other tissues during CR, and would have the sum effect of increasing SirT1 activity (Cohen et al., 2004; Chen et al., 2008). Given the data presented in Chapters 3 and 4, SirT1 activation would be predicted to promote the differentiation of osteoblasts and repress the differentiation of osteoclasts. It is therefore intriguing that this is exactly the profile that we observe in CR mice.

It is also interesting that SirT1 knockout mice display a phenotype that is the direct opposite of CR mice, namely where bone mass is reduced due to an increase in osteoclast numbers and a decrease in osteoblast numbers. To determine whether SirT1 is genetically downstream of CR, we performed CR on whole-body SirT1 knockout mice. Importantly, we find that knockout mice fail to show an increase in bone mass during CR. We are currently performing histology to determine whether SirT1 knockouts also fail to show the changes in osteoblast and osteoclast numbers associated with CR. If they do, it would provide strong evidence indicating SirT1 acts downstream of CR.

One question that remains unanswered is which tissues require SirT1 in order to mediate these effects. Given SirT1's cell autonomous role in osteoblast and osteoclast differentiation, these two cell types represent ideal candidates. Therefore, CR studies will have to be repeated with the osteoblast and osteoclast specific knockouts described in earlier chapters. However, recent evidence suggests that SirT1 might also act in other tissues to influence bone mass. For instance, SirT1 knockout mice fail to show an increase in activity upon CR (Chen et al., 2005). Since physical activity is associated with increased bone mass, SirT1's regulation of this response might also affect bone indirectly. Further, SirT1 is also a known repressor of PPAR γ , a

negative regulator of osteoblast commitment and bone mass (Picard et al., 2004). SirT1 might therefore regulate bone mass by also increasing osteoprogenitor numbers by promoting the commitment of the early MSC towards the osteoblast lineage. Ultimately, the only way to distinguish between these disparate effects will be to perform CR on different SirT1 tissue specific knockouts.

In summary, we present evidence that SirT1 might is a novel nutrient sensitive regulator of bone remodeling *in vivo*. It is interesting to note that activation of SirT1 in another context, namely with resveratrol, has also been shown to lead to increases in bone mass (Pearson et al., 2008). Since resveratrol treatment mimics many aspects of CR, it is noteworthy that both interventions result in positive gains in bone mass. It will be interesting to determine whether osteoblast and osteoclast counts change in resveratrol treated animals as well, and whether resveratrol's effects are abrogated in SirT1 knockout animals. Finally, given these findings, we propose that CR mimetics might represent a new class of drugs for the treatment of osteoporosis.

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Figure 1. Calorie restriction (CR) results in increases in bone mass.

(A) Mice (from the National Institutes of Aging) were placed on a CR diet (40% below ad libitum) for 8 months, starting at 4 months of age. CR mice show an expected decrease in body weight compared to ad libitum (AL) fed controls.

(B) Micro-CT scans reveal a clear increase in the bone mass of CR mice, including a 38% increase in bone volume/total volume (BV/TV) and a 32% increase in bone mineral density (BMD).

Error bars represent SEM. (n = 7 for each group; * p<.05; ** p<.01; *** p<.005).

Figure 1

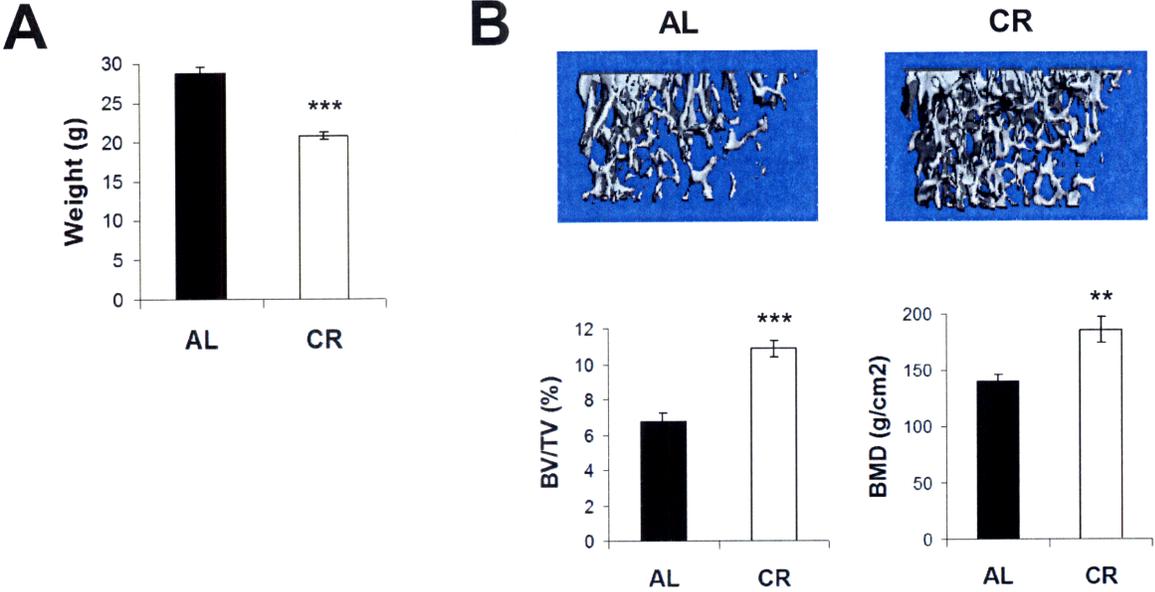


Figure 2. CR increases osteoblast numbers and decreases osteoclast numbers.

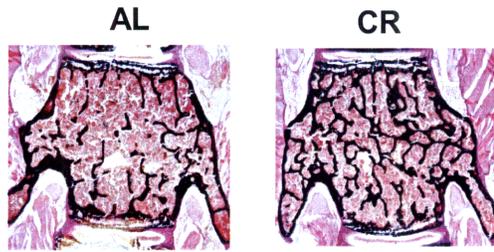
(A) Gross histology shows a clear increase in the amount of mineralized bone occupying the L3-L4 vertebrae of CR mice (as assayed by Von Kossa staining; black indicates mineralized bone).

(B) Histomorphometry shows that the increase in bone volume of CR mice is accompanied by a decrease in osteoclast numbers per bone surface (Oc. S./BS) and an increase in osteoblast numbers per bone surface (Ob. S./BS).

Error bars represent SEM. ($n \geq 7$ for each group; * $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 2

A



B

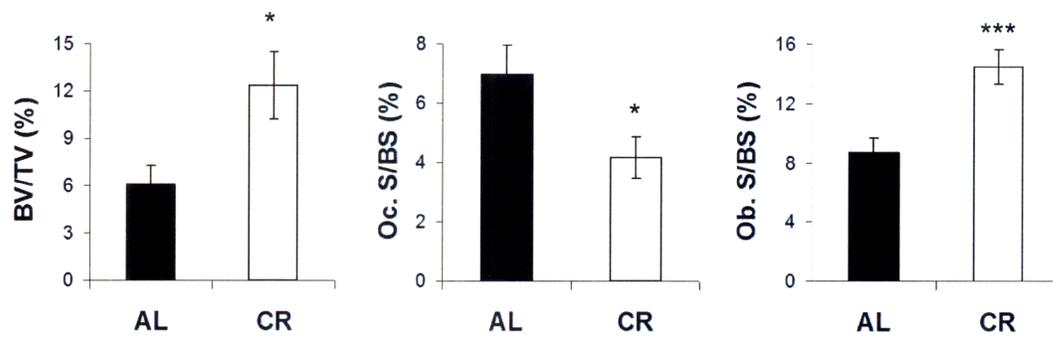


Figure 3. SirT1 expression and activity are induced upon CR.

(A) SirT1 expression is increased approximately two fold in the calvaria of CR mice.

(B) Calvaria of CR mice also show a trend towards increased NAD/NADH levels, suggesting activation of SirT1 enzymatic activity.

Error bars represent SEM. ($n \geq 3$ for each group; $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 3

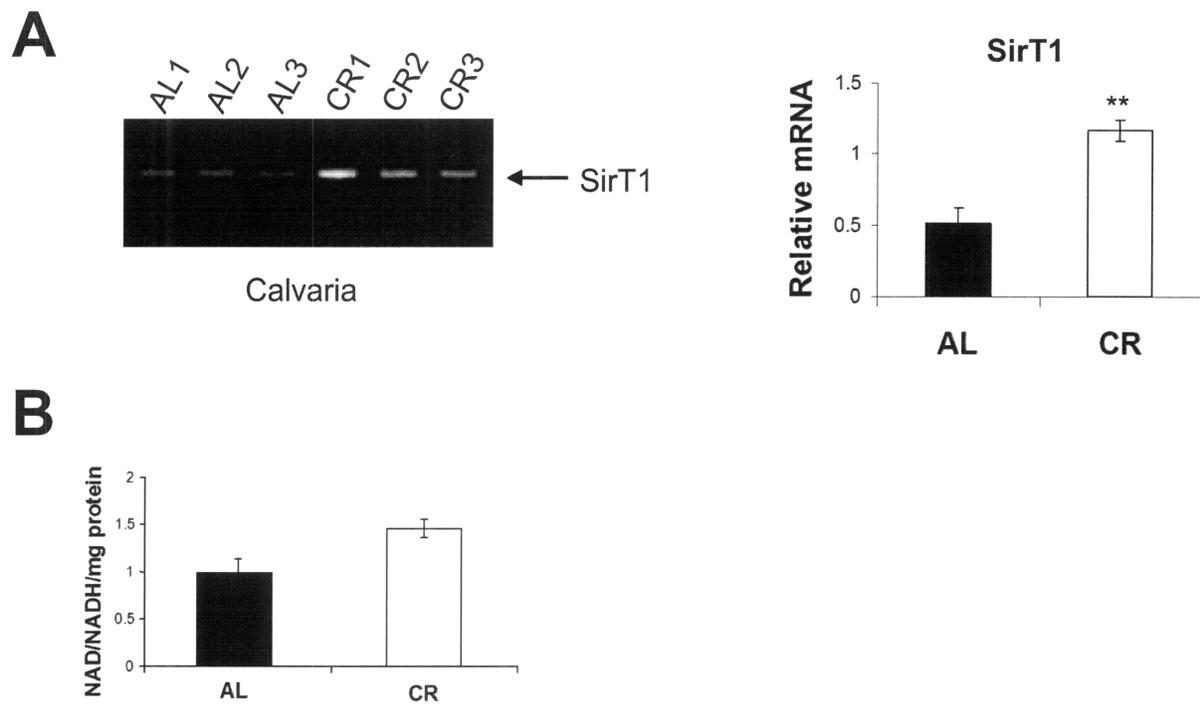


Figure 4. SirT1 whole-body knockouts fail to display increases in bone mass upon CR.

(A) 12 month old wildtype or knockout mice were placed on CR (35% below ad libitum levels) for 4 months and weighed weekly.

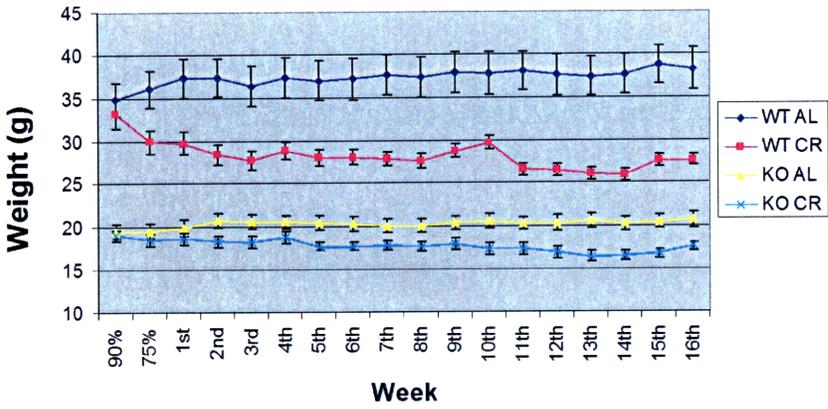
(B) Upon completion of the regimen, both wildtype and knockout CR mice showed a significant decrease in body weight. Knockout mice have previously been shown to lose less weight upon CR since they are leaner to begin with (Chen et al., 2005).

(C) Micro-CT shows that wildtype mice display an increase in bone mass in response to CR, while knockout mice do not.

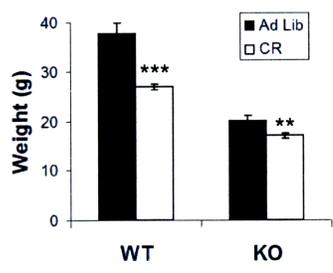
Error bars represent SEM. ($n \geq 6$ for each group; * $p < .05$; ** $p < .01$; *** $p < .005$).

Figure 4

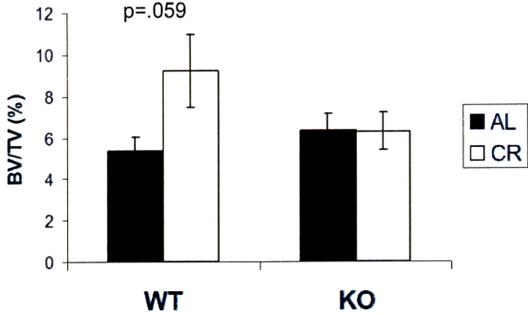
A



B



C



Chapter 6

Conclusions

SirT1 - a novel nutrient sensitive factor linking metabolism to bone remodeling and osteoporosis?

Osteoporosis occurs ubiquitously with aging and is characterized by either an increase in osteoclasts or a decrease in osteoblasts (Harada and Rodan, 2003). This leads to an imbalance in bone remodeling, whereby either too much bone is resorbed (Type I, post-menopausal osteoporosis) or too little bone is formed (Type II, age-related osteoporosis). Interestingly, SirT1 whole-body knockouts display characteristics resembling both types of osteoporosis, suggesting a role for SirT1 in both osteoblasts and osteoclasts. To determine whether this action was through cell autonomous or non-cell autonomous means, we constructed mice that had SirT1 deleted specifically in either osteoblasts (ObKOs) or osteoclasts (OcKOs). Both mice display an osteoporotic phenotype, which in the ObKOs is associated with decreased osteoblasts, and in the OcKOs with increased osteoclasts. Therefore, we conclude that SirT1 regulates bone mass *in vivo*, by cell autonomously regulating osteoblast and osteoclast differentiation.

SirT1 appears to exert its effects in two ways: by promoting osteoblastogenesis via Runx2 activation and repressing osteoclastogenesis via NF κ B inhibition. Although SirT1 has previously been shown to repress NF κ B in a number of different contexts, this is the first instance showing that this repression is important for osteoclastogenesis (Fulco et al., 2003; Pfluger et al., 2008). NF κ B activation is, in fact, one of the pivotal steps in osteoclasts differentiation: RankL binding to the Rank receptor, results in NF κ B translocation to the nucleus and transcriptional activation of NFATc1, a master osteoclast transcription factor (Asagiri and Takayanagi, 2007). It is therefore notable that we observe hyperinduction of NFATc1 in knockout cells, which alone has been shown to be sufficient for osteoclast differentiation (Takayanagi et al., 2002; Ishida et al., 2002). Since these effects can be rescued in knockout cells

via chemical inhibition of NF κ B, we conclude that SirT1 normally represses osteoclastogenesis by inhibiting NF κ B.

We also present evidence that SirT1 interacts with and regulates Runx2 activity in osteoblasts. Unlike other HDACs, SirT1 acts to enhance Runx2 activity, thereby promoting osteoblast differentiation (Jensen et al., 2007). Since Sirtuins are unique among other HDACs in that their function is linked to metabolism, these findings present a novel link between nutrient conditions and bone remodeling. Further studies will be required to confirm this, as well as how exactly SirT1 promotes Runx2 activity.

Interestingly, the osteoporotic phenotype in both the ObKOs and OcKOs appear more severe in females than in males. The reasons behind this are yet unclear, though it has been suggested that SirT1 acts downstream of the estrogen signaling pathway (Elbaz et al., 2009). Further, the onset and progression of the osteoporotic phenotype differs in the three knockout models. Whole-body knockouts (WBKOs) display a low bone mass phenotype at 1 month of age that appears to worsen as they age, while ObKOs show osteoporosis only at 4 months of age. The reasons behind this are unclear, though this delay suggests a role for SirT1 specifically in bone remodeling, perhaps downstream of a hormone signaling pathway. OcKOs, on the other hands, show osteoporosis both at 1 month and 4 months, though the phenotype does not appear to worsen with age. These data combined, suggest that the bone deficiencies observed in 1 month WBKOs is likely due to hyperactivity of osteoclasts, while the worsening of the phenotype with age results from hypoactivity of osteoblasts. However, preliminary analysis of double-knockout (DKO) mice (containing deletion of SirT1 in both osteoblasts and osteoclasts) at 4 months of age does not show a synergistic effect, suggesting additional non-cell autonomous effects in WBKOs (**Figure 1**). Further studies will therefore be required to determine whether DKOs show

increased bone loss with advanced age or whether expression of Cre in the DKO mice is comparable to the single osteoblast or osteoclast knockouts.

As mentioned before, the use of NAD as a co-factor has put the Sirtuins at the nexus of metabolism and transcriptional regulation (Haigis and Guarente, 2006). SirT1 specifically has been implicated as a possible mediator of CR for the following reasons: 1) SirT1 expression is induced in certain tissues upon CR (Cohen et al., 2004); 2) SirT1 activity is increased in certain tissues due to an increase in NAD/NADH levels (Chen et al., 2008); 3) SirT1 knockout mice fail to display certain attributes of CR, including increased activity, upregulation of metabolism, and an increase in lifespan (Chen et al., 2005; Boily et al., 2008). We were therefore interested to examine how CR influenced bone mass. Surprisingly, we found that long-term CR of adult mice led to striking gains in bone mass. Further, even short term CR (4 months) was sufficient to increase bone mass in older mice (12 months old). Importantly, SirT1 expression and activity is induced nearly two fold in the calvaria of CR mice. Based on data presented in earlier chapters, activation of SirT1 would be predicted to promote osteoblastogenesis and repress osteoclastogenesis, which would result in increases in bone mass. This is in fact precisely what we observe with CR. SirT1 might therefore constitute a novel nutrient sensitive factor linking metabolism to bone remodeling, and possibly osteoporosis.

Intriguingly, the phenotype of the CR mice is the exact opposite of the whole-body SirT1 knockout mice. To determine whether CR acted through SirT1, we placed a cohort of WBKO mice on CR for 4 months. While wildtype mice show an expected increase in bone mass upon CR, WBKO mice show no such gains. This finding therefore suggests that SirT1 might act downstream of CR. We are currently performing histology to determine whether WBKO mice also fail to show the expected changes in osteoblast and osteoclast counts.

Preliminary analysis of CR performed in 3 month old ObKOs, OcKOs, or DKOs shows that CR has less dramatic effects on bone when performed early in life (**Figure 2**). Further, it appears that all of the conditional knockout mice appear to respond to CR as well as wildtype mice, suggesting that SirT1 is not required cell autonomously in osteoblasts or osteoclasts at young age. However, these studies will be needed to be repeated in a larger cohort of animals at an older age (i.e. 12 months) or for a longer duration of CR (i.e. 8 months) in order to make any definitive conclusions. However, the different strain backgrounds of knockout models complicates any direct comparisons.

In summary, we show that SirT1 is an important regulator of bone mass *in vivo*. Given its regulation of osteoblast and osteoclast differentiation, SirT1 provides a unique pharmacological target for the treatment of both post-menopausal and age-related osteoporosis (**Figure 3**). This has rather important implications, since there are currently no effective treatments for age-related osteoporosis. In this regard, it is rather intriguing that a recent report shows mice treated with the SirT1 agonist, resveratrol, show preserved bone mass with aging (Pearson et al., 2008). Our analysis reveals that this protection is associated with hyperactivation of Runx2, suggesting it is at least in part mediated by stimulating osteoblast activity. Based on its repression of PPAR γ , SirT1 would also be predicted to promote the earlier osteoblast commitment step of mesenchymal stem cells. Whether this acts to counter the observed increases in adipogenesis at the expense of osteoblastogenesis during aging is an exciting possibility. Lastly, our findings indicate that SirT1 agonists would also act to repress osteoclastogenesis, though this also needs to be confirmed experimentally.

Not surprisingly, it is currently an exciting time in Sirtuin biology. The discovery of a novel set of more potent and specific activators (and inhibitors) of SirT1 allow *in vivo*

modulation of SirT1 activity within relatively easy reach (Milne et al., 2008). Therefore, it will be particularly interesting to examine how pharmacological activation or inhibition of SirT1 affects bone (and other tissues), particularly during aging. We have already shown that one of these agonists, SRT2813, leads to increased differentiation of primary osteoblasts through activation of Runx2. We therefore patiently (and optimistically) wait to see whether these CR mimetics prove efficacious in the battle against osteoporosis.

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Figure 1. SirT1 double-knockout mice (DKOs).

Mice containing deletion of SirT1 in both osteoclasts and osteoblasts (DKOs) show reduced bone mass at 4 months of age, though not in an additive manner as expected nor to the same extent as whole-body SirT1 knockout mice.

Error bars represent SEM. (n \geq 6 for each group; * p<.05; ** p<.01; *** p<.005).

Figure 1

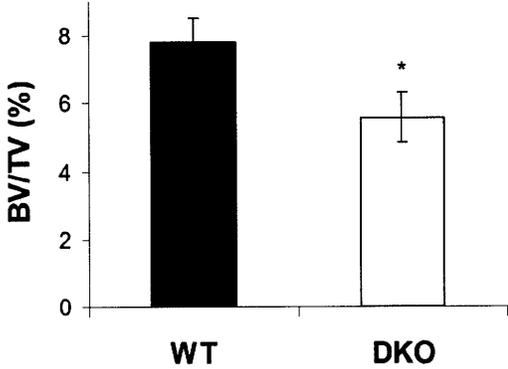


Figure 2. The effect of calorie restriction (CR) on osteoblast (ObKO), osteoclast (OcKO), or double-knockout (DKO) mice.

Mice were placed on a CR diet (35% below ad libitum) starting at 3 months of age and maintained for 4 months. Micro-CT analysis shows that CR leads to modest increases in bone mass in all four groups of mice.

Error bars represent SEM. ($n \geq 4$ for each group).

Figure 2

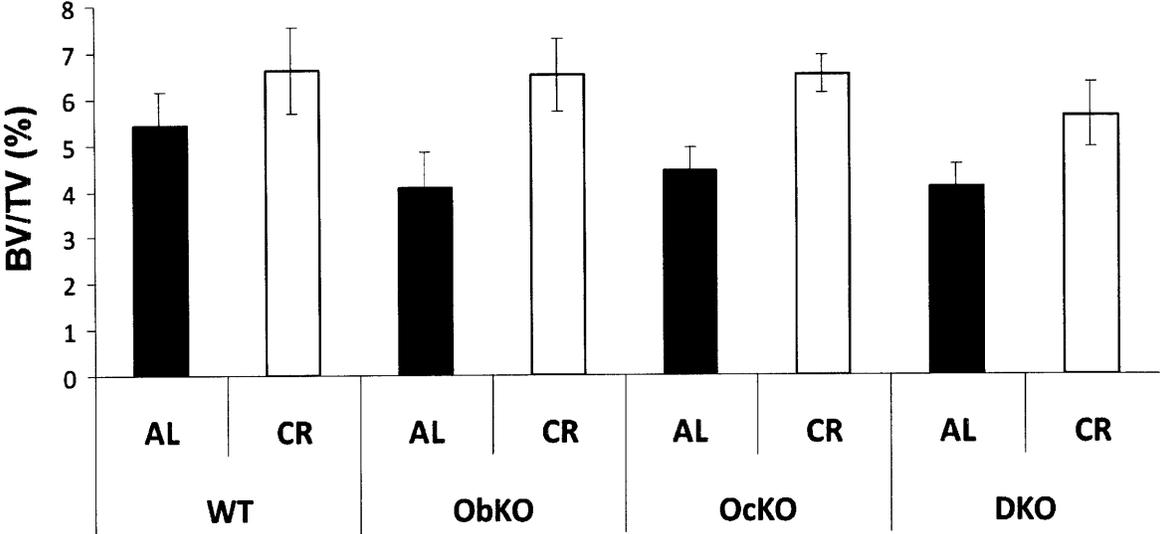


Figure 3. A model for the effects of SirT1 on bone.

SirT1 normally regulates bone homeostasis by repressing osteoclastogenesis via inhibition of NF κ B signaling and promoting osteoblastogenesis through activation of Runx2. Our data suggest that induction of SirT1 during CR might help promote bone mass by favoring bone formation over resorption, thereby countering the effects of osteoporosis during aging. Further, based on its known repression of PPAR γ , SirT1 would be predicted to also promote osteoblast commitment of the early mesenchymal stem cell (MSC) progenitor.

Figure 3

